Inverted repeat sequences in the genome of *Rhodomicrobium vannielii*

by

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Contents

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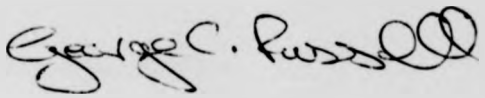
This thesis was typed on a BBC model B microcomputer with "Wordwise" word processing facility and was printed on an Epson FX-80 dot-matrix printer.

Declaration

The work contained in this thesis was the result of original research conducted by myself under the supervision of Dr. N.H. Mann. All sources of information have been specifically acknowledged by way of reference.

None of the work contained in this thesis has been used in any previous application for a degree



George C. Russell

Dedication

To my family,

who make all things possible.

".....if we have learned one thing from the history of invention and discovery, it is that in the long run - and often in the short one - the most daring prophecies turn out to be laughably conservative."

Arthur C. Clarke

from "The conquest of space"

Abbreviations

Ap	ampicillin, sodium salt
ATP	adenosine triphosphate
bp	base pairs
BSA	Bovine Serum Albumin
Ci	Curie (3.7×10^{12}DPM)
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DFP	Diisopropylfluorophosphate
DMSO	dimethyl sulphoxide
DNAse	deoxyribonuclease
DPM	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid, disodium salt
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	hour
kb	kilobase pairs
kD	kilodaltons
mA	Amperes $\times 10^{-3}$
Mr	molecular weight
O.D.	optical density, absorbance
PMSF	phenylmethylsulphonylfluoride

PPO	2,5-diphenyloxazole
psi	pounds per square inch
RNAse	ribonuclease
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
Tc	tetracycline
TCA	trichloroacetic acid
TEMED	N,N,N,N'tetramethylenediamine
Tris	hydroxymethylaminomethane
Triton X-100	octyl phenoxy polyethoxyethanol
α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
λ	lambda
ω	omega
φ	phi
σ	sigma
μ	mu

Summary

In this work the abundance, structure, distribution and possible role of the inverted repeat sequence DNA (IR DNA) of *R. vannielii* was investigated.

Approximately 7% of the *R. vannielii* genome was found to exist as inverted repeat sequences by nuclease S1 digestion of heat-denatured, rapidly renatured DNA; this compared with 3% for *E. coli* K12 DNA. The inverted repeat sequence DNA of *R. vannielii* formed two size classes: a heterogeneous high molecular weight class with a size range of 100-700 base pairs (bp), and a low molecular weight class comprised of fragments of 17 and 27bp.

The genomic distribution of these two inverted repeat classes was investigated by DNA/DNA hybridization studies. *In vitro* ^{32}P-labelled IR DNA was used to probe Southern blots of *Eco*RI and *Hind*III restriction enzyme digests of *R. vannielii* DNA. Both classes of inverted repeat DNA showed hybridization with many bands throughout the restriction digests, suggesting that the sequences were not clustered but were dispersed throughout the genome. Low molecular weight IR DNA however, hybridized to four specific bands in an *Hae*III digest of *R. vannielii* DNA suggesting that this enzyme could reveal some repetitive structure in the genome which the other enzymes used could not. Low molecular weight IR DNA was also found to hybridize throughout the high molecular weight IR DNA class, indicating that the two IR DNA classes share some sequences and may be derived from the same chromosomal loci. Competition filter-binding assays, designed to detect protein-DNA interactions, showed that about 8% of high molecular weight IR DNA sequences appeared to be bound specifically by *R. vannielii* protein while low molecular weight IR DNA was not bound.

The genomic plasticity of *R. vannielii* was investigated by hybridization and by a dual-labelling method. Although these experiments gave inconclusive results there were indications that sequence rearrangements might occur during *R. vannielii* swarmer cell differentiation.

Attempts to clone IR DNA directly by two methods had only limited success because of difficulties in the identification of IR DNA-containing recombinant plasmids and suggested that cloning of IR DNA should be done by hybridization screening of chromosomal gene libraries.

The serine hydrolases of the differentiating swarmer cell were also examined. Using an inhibitor labelling technique up to eight bands could be identified on fluorographs of SDS gradient gels and a number of changes in banding were observed to occur during swarmer cell differentiation.

Chapter 1 Introduction

1.1 General Introduction

One of the most active areas of research in molecular biology is the investigation of the mechanisms by which gene expression is controlled. In this field bacteria have been widely used because of their relative simplicity, their amenability to research and the biochemical and genetical background knowledge which exists. The organism studied in this work is the photoheterotrophic bacterium Rhodomicrobium vannielii. This bacterium possesses an obligate differentiation event as part of its cell cycle and may be used as a model system for bacterial differentiation.

Whilst the mechanisms of control of gene expression most easily studied are those in which some simple biochemical or molecular event can be observed, a higher order of control is found in the cell division cycle in which cell growth and division are coordinated with DNA replication by spatially and temporally controlled gene expression. The best studied example of this is the cell division cycle of E. coli, but even here the underlying control mechanisms are not yet thoroughly understood.

The same coordination of gene expression is also apparent in prokaryotic differentiation during which a fixed programme of changes in gene expression is observed. Such programmes may be initiated as part of the cell division cycle or in response to environmental conditions, and they are a potentially rich source of information on how the coordination of gene expression may be achieved. Some of

the best characterised examples of prokaryotic cell cycles and differentiation are described in section 1.2. Wherever possible the mechanisms of control of gene expression found or thought to occur will be presented. Multicellular prokaryotic growth and development will not be discussed as they are outside the scope of this work.

Section 1.3 contains a discussion of the possible role of inverted repeat sequences in control of gene expression and prokaryotic differentiation. These sequences are involved in most DNA rearrangements and are important in regulatory processes. They also appear to be much more abundant in bacteria which exhibit differentiation compared to those which possess a simple vegetative cell cycle. Thus, the investigation of these sequences in differentiating bacteria may provide useful information about the coordination and control of the differentiation process. This work is an investigation of the inverted repeat sequence DNA of Rhodomicrobium vannielii; its abundance, structure, distribution and role in differentiation.

1.2 Prokaryotic Morphogenesis and Differentiation

1.2.1 Introduction and Definitions

Any description of the prokaryotic cell cycle or prokaryotic differentiation will necessarily use a number of specialized terms. In this work the definitions proposed by Whittenbury and Dow (1977) will be used as follows. Morphogenesis: changes in the shape or size of a cell or

components thereof.

Differentiation: the morphogenetic change of one cell type to form another, distinct cell type (differentiation is therefore a specialized form of morphogenesis).

Development: the differentiation of a cell in response to intracellular conditions, sometimes the result of intercellular interactions.

Cell cycle: that series of events involved in DNA replication and cell division occurring in a temporally controlled cyclic manner.

Monomorphic cell cycle: a cell cycle in which a single morphological cell type is observed throughout. e.g. E. coli

Dimorphic cell cycle: a cell cycle in which two distinct morphological forms are distinguishable, differences normally being apparent at cell division. e.g. Caulobacter spp.

Polymorphic cell cycle: a cell cycle in which two or more cell morphologies may be observed, possibly under different nutrient conditions, each of which undergoes its own cell cycle. e.g. Arthrobacter spp., Rhodomicrobium vannielii

Amongst prokaryotes there are widely varying degrees of organisation and intercellular cooperation. The majority of prokaryotes exhibit unicellular organisation, each cell being physically and physiologically independent of other cells of its species, but other forms of organisation are found. Some prokaryotes exhibit colonial organisation in which the cells are physically connected, but are apparently independent physiologically. e.g. Rhodomicrobium vannielii, Hyphomicrobium spp.. Multicellular organisation is also found in which there may be both structural and

physiological interdependence between the cells. Intercellular interactions determine the development of cells and cooperation between cells occurs. e.g. heterocyst-forming cyanobacteria; Myxococcus spp.

Some of these concepts will be expanded on in the following examples of cell cycle types and control.

1.2.2 The Cell Division Cycle of Escherichia coli

1.2.2.1 Introduction

Early work on the monomorphic cell cycle of Escherichia coli (figure 1.1) led to its description in terms of the growth parameters I,C and D (Cooper & Helmstetter, 1968; Helmstetter et al., 1968), which are defined below.

I: The period of time required for accumulation of sufficient "initiation potential" to allow a single initiation of DNA replication. i.e. the time between succeeding initiations.

C: The time necessary for replication of the chromosome.

D: The time between termination of DNA replication and cell division.

I is always equal to generation time as suggested by Maaloe and Kjeldgaard (1966), and is inversely proportional to the growth rate of the culture. C and D are fixed at high growth rates (generation times less than about 70 minutes) and increase with generation time at lower growth rates (Helmstetter, 1969). The relationship between I, C and D at various growth rates is shown diagramatically in figure 1.2. Figure 1.2b illustrates that at longer

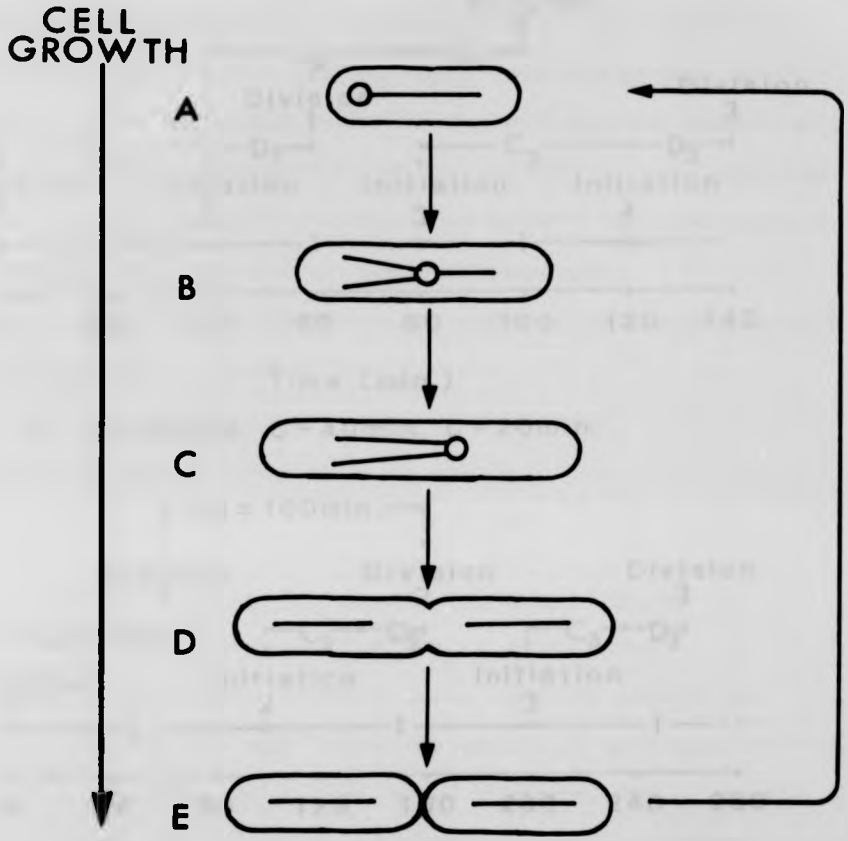
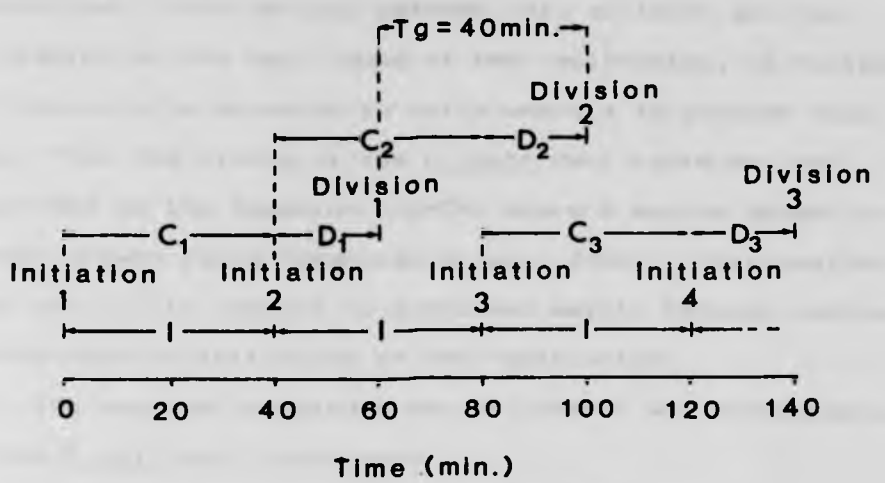


Figure 1.1 Schematic illustration of the E. coli cell cycle at low growth rate. A: Initiation of DNA replication. B: DNA replication. C: Termination of DNA replication. D: Segregation and septation. E: Cell separation.

———Linear representation of genome

O Active replication complex

a. I = 40min. C = 40min. D = 20min.



b. I = 100min. C = 40min. D = 20min.

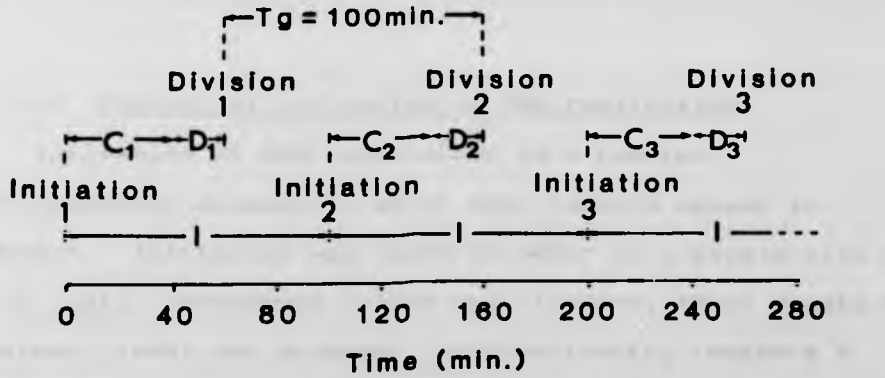


Figure 1.2 Relationship between cell cycle parameters and generation time (Tg) of *E. coli* at different growth rates.

generation times a fourth growth parameter can be identified. This period, between cell division and the initiation of the next round of DNA replication, is called B and can only be observed in cells where I is greater than C+D. Thus the timing of the E. coli cell cycle may be described by the equation I=B+C+D where B may be absent at higher growth rates (Skarstad et al., 1983). This implies that cell cycle control is exercised mainly through control of the rate of initiation of DNA replication.

The two most studied areas of control and coordination of the E coli cell cycle are:

1. Control of initiation of DNA replication.
2. Control of cell division.

1.2.2.2 Control of initiation of DNA replication

Initiation of DNA replication is a complex, multicomponent process in which many factors appear to interact. Initiation was found to occur at a single site on the E. coli chromosome called oriC (Cairns, 1963; Nagata & Meselson, 1968) and proceeds bidirectionally (Masters & Broda, 1971). oriC was cloned in plasmid pBR322 and its minimum size and content determined (Oka et al., 1980). Initiation also requires RNA synthesis at or just prior to initiation (Lark, 1972) and the action of specific gene products (Wechsler & Gross, 1971; Carl, 1970; Beyersmann et al., 1974; Wada & Yura, 1974)

Models for control of initiation were based either on the inhibition of initiation when it was not required or on the stimulation of initiation when it was required (Pritchard et al., 1969; Sompayrac & Maaloe, 1973). The

main requirement for any model however, was that initiation was coupled to cell growth rate.

Although the molecular events involved in control of initiation are not yet known, many of the requirements for, and processes in, initiation have been elucidated. Much of this work is consistent with the suggestion that the rate of initiation of DNA replication is regulated by the rate of completion of an "initiation complex" which may be membrane bound and the synthesis of which is correlated with growth rate. This initiation complex is probably bound to the chromosome at oriC (Hendrickson et al., 1981) and its completion promotes or facilitates transcription of initiation specific RNA from the supercoiled oriC region (Tippe-schindler et al.,1979; Filutowicz, 1980; Filutowicz & Jonczyk, 1981). This RNA then acts as a primer for DNA replication (Lother & Messer, 1981). Translation of an origin-proximal transcript produces a repressor of initiation (Eberle et al., 1982) which may act directly at oriC or indirectly by interfering with formation of new initiation complex. Immediate reinitiation may also be prevented by changes in supercoiling of the origin region during and shortly after its replication (Helmstetter & Krajewski, 1982).

1.2.2.3 Control of cell division

Cell division is tightly linked to DNA replication but does not appear to be dependent on initiation of DNA replication for its timing. Instead, cell division is dependent on completion of a round of DNA replication (Clark, 1968) and any inhibition of DNA synthesis delays the

following cell division for the same period (Helmstetter & Pierucci, 1968). Two distinct periods of protein synthesis are required for cell division. The first, normally concurrent with DNA replication (Pierucci & Helmstetter, 1969), may allow the accumulation of necessary division proteins - some of which appear to be heat labile (Smith & Pardee, 1970). The second period of protein synthesis is much shorter and is required between termination of DNA replication and cell division (Jones & Donachie, 1973) suggesting a requirement for a specific "termination protein" to allow normal cell division.

Models for the control of cell division and its coupling to DNA replication fall into two main classes:

1. Positive control models which suggest that the accumulation of required factors for division in the correct proportions and at the appropriate site trigger division, and that the synthesis of these factors is coupled to, or regulated by DNA replication (Jones & Donachie, 1973; Zaritsky & Pritchard, 1973).
2. Negative control models which require that an inhibitor of cell division represses premature division and that perturbations of DNA replication, or DNA damage, induce high levels of this inhibitor, preventing cell division until DNA repair has been accomplished (Witkin, 1967).

The first genetic evidence for coupling of DNA replication with cell division came with the characterisation of lon mutants (Walker & Pardee, 1968). Mutants of this type were highly sensitive to ultraviolet light, responding to minute doses of UV irradiation with complete inhibition of cell division. Cell mass continued

to increase normally however, resulting in the formation of filamentous, multinucleate cells which were inviable unless cell division resumed. The behaviour of these mutants gave further credence to negative control models for regulation of cell division. Other loci have been found, mutations in which, restored normal cell division in filament forming mutants including lon mutants (George et al., 1975). The gene product of one of these loci, sfiA (called sulA by some workers), has since been characterised as an inducible repressor of cell division, synthesised at high levels after perturbations of DNA replication (Huisman & D'Ari, 1981). The sfiA product inhibits cell division by inhibition or inactivation of the ftsZ gene product (Lutkenhaus, 1983; Ward & Lutkenhaus, 1984), blocking an essential step in cell division which may be initiation of septum formation (Walker et al., 1975). It does not appear however, to be involved in the normal regulation of cell division, but is part of the "SOS" response to DNA damage. A second pathway of division inhibition, independent of sfiA, seems to be in operation and this pathway may be part of the normal mechanism of regulation of cell division (Burton & Holland, 1983).

Positive regulation of cell division may also occur. The ftsA gene product is synthesised in a ten minute burst immediately before septation (Donachie et al., 1979) and is required in an active form throughout the process of septation. A short period of DNA synthesis appears to be necessary for the accumulation or activity of the ftsA protein (Tormo et al., 1980). The ftsA product is therefore a likely candidate for the termination protein suggested by

Jones and Donachie (1973) which is produced at termination of DNA replication and allows or promotes cell division at a fixed time thereafter.

It is possible then, that cell division is controlled by the accumulation of necessary factors, including the structural proteins of the division septum; the termination of the oldest round of DNA replication, which promotes or permits *fts*A transcription; the absence or inactivation of inhibitory proteins like the *sfi*A protein; and the activation of a "septation complex" by *fts*A protein leading to septation and cell division. The septation complex is probably an accumulation of periplasmic and inner and outer membrane proteins which interact with each other and with the cell wall to produce the coordinated invagination seen at septation.

Control of the *E. coli* cell cycle is a complex process involving many components. Regulation and coordination appears to be acheived by:

1. Linking initiation of DNA replication to growth rate either by accumulation of initiation-specific proteins or by dilution or inactivation of an inhibitor of initiation.
2. Accumulation of necessary division proteins during DNA replication, probably in proportion to growth rate.
3. Synthesis of protein(s) at termination of DNA replication which initiates and maintains the septation process.
4. Inhibition of premature division by specific inhibitors synthesised in response to DNA damage or other perturbations of chromosome replication.

1.2.3 Bacillus subtilis Cell Cycle and Differentiation

1.2.3.1 Introduction

Bacillus subtilis is a Gram positive, aerobic bacterium with a dimorphic cell cycle. Vegetative growth proceeds in a pattern similar to that observed for E. coli (Paulton, 1971), although cell division and DNA replication do not appear to be tightly coupled (Donachie et al., 1971). During the late exponential phase of batch growth however, as the culture medium becomes exhausted, dormant, highly resistant endospores are produced which can germinate and outgrow under suitable conditions. The cell cycle of B. subtilis is illustrated simply in figure 1.3.

1.2.3.2 Initiation of sporulation

Experiments to study sporulation of B. subtilis in continuous culture suggested that sporulation could only be initiated at a specific stage in the chromosome replication cycle (Dawes et al., 1971). This stage, about 15 minutes after initiation of replication, may correspond with the passage of a replication fork through a sporulation specific locus (Mandelstam & Higgs, 1974; Dunn et al., 1978). Transfer of cells into poor medium during DNA replication leads to the formation of a nucleoid which appears diffuse and filamentous by electron microscopy, like that found in stage I of sporulation. Cells growing in rich medium have nucleoids with a very different appearance: compact with well defined shape and position (Dawes et al., 1971). A model for the cell cycle regulation of initiation of sporulation based on these observations is illustrated in

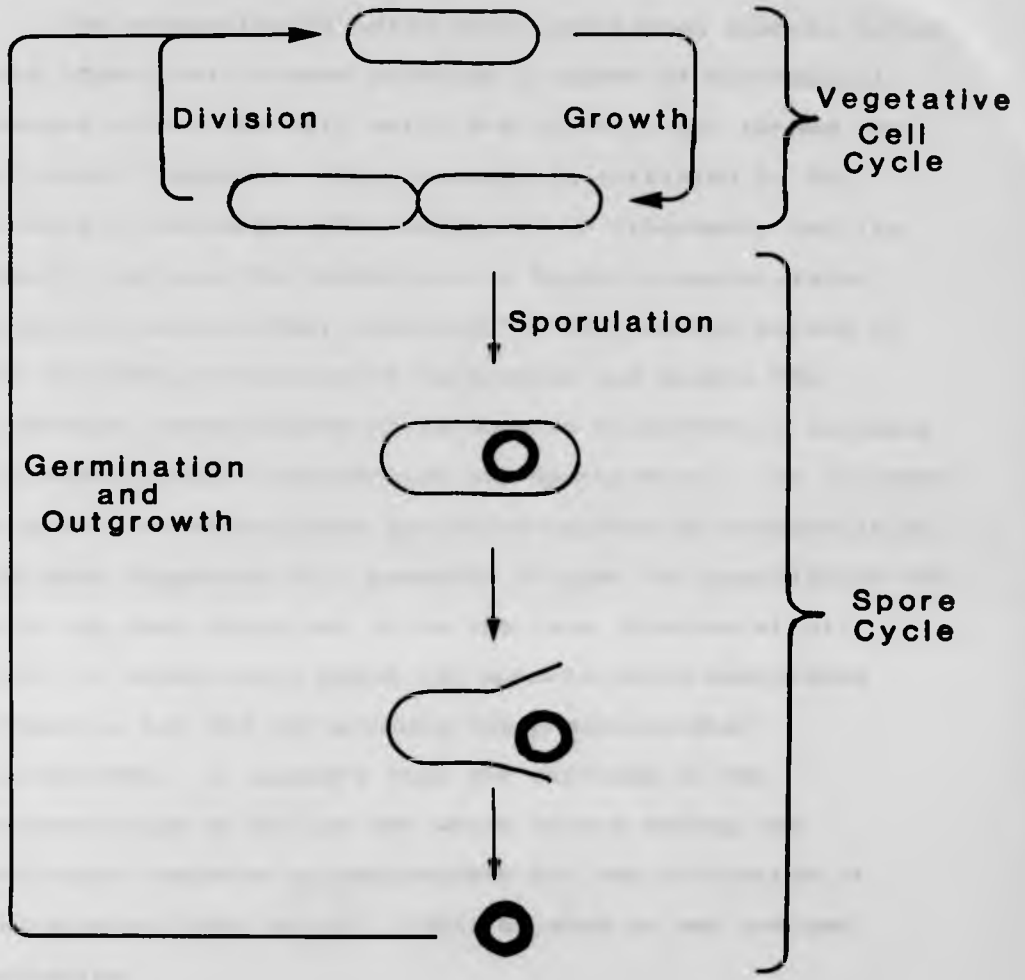


Figure 1.3 The dimorphic cell cycle of B. subtilis

figure 1.4.

The exhaustion of amino acids and carbon sources during late logarithmic growth produces a number of biochemical changes within the cell which are collectively termed the stringent response. This response is initiated by the binding of uncharged tRNA molecules to ribosomes, and its results include the production of highly phosphorylated guanosine nucleotides; reduction in the concentrations of GTP and GDP; inhibition of nucleotide and stable RNA synthesis; derepression of amino acid biosynthetic enzymes; increased protein degradation and sporulation. The increase in highly phosphorylated guanosine nucleotide concentration has been suggested as a possible trigger for sporulation but this has been shown not to be the case (Nishino et al., 1979) in experiments using rel mutants which sporulated normally, but did not produce the phosphorylated nucleotides. It appears that the decrease in the concentration of GTP or GDP which occurs during the stringent response is responsible for the initiation of sporulation (Ochi et al., 1982) by some as yet unknown mechanism.

1.2.3.3 Morphology and biochemistry of sporulation

Once initiated, endospore formation proceeds through a temporally ordered series of morphological changes. These morpholgical changes are associated with well defined biochemical events which are used as markers in sporulation (for reviews see Piggot and Coote (1976) and Hanson (1979)). The morphological changes observed during sporulation are illustrated in figure 1.5.

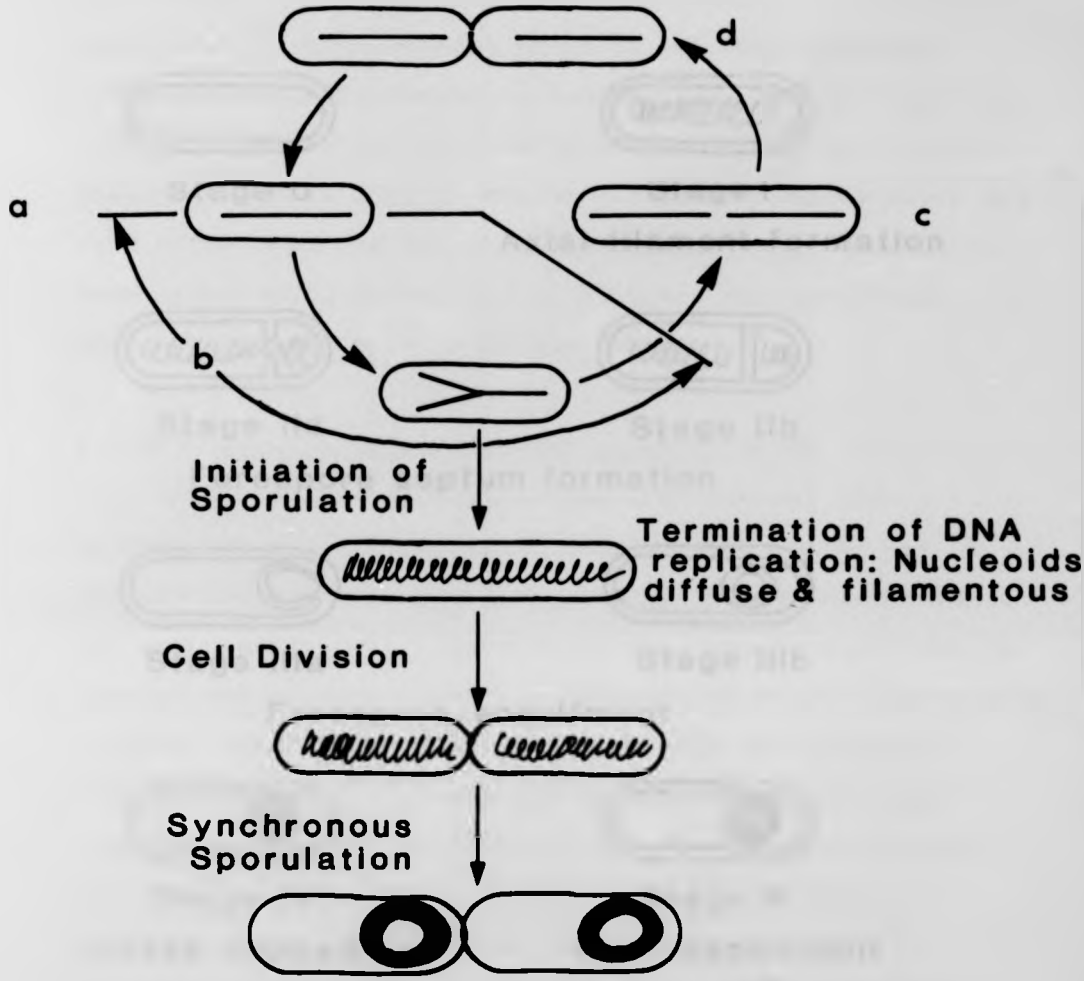


Figure 1.4 Initiation of sporulation in the cell cycle of B. subtilis. a. Initiation of DNA replication. b. That period of the cell cycle during which cells are competent to initiate sporulation. c. Termination of DNA replication, nucleoids compact and separate. d. Cell division.

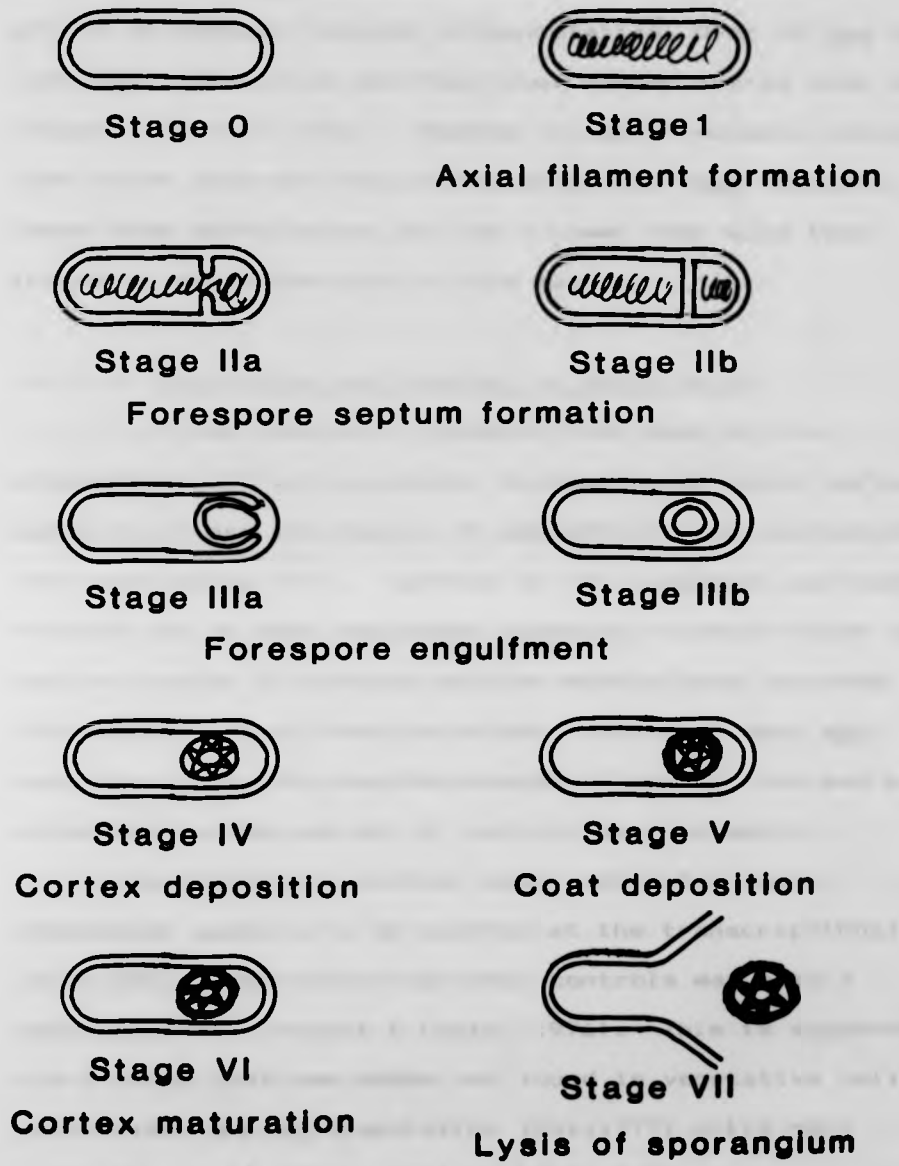


Figure 1.5 Morphological stages of endospore formation (after Hanson et al., 1979).

Most research on sporulation involves the use of asporogenous (spo) mutants which do not sporulate, but arrest at specific stages in sporulation. Over 30 spo loci have been identified and the total number may be over 40 (Piggot & Coote, 1976). Another class of mutants which are less often used are the oligosporogenous (osp) mutants. These show sporulation, but at a lower than wild type frequency characteristic of the mutant.

1.2.3.4 Regulation and control of sporulation

It is now generally accepted that most of the biochemical and morphological changes which occur during sporulation are the result of sequential gene expression in the sporulating cell. Control of this sequence may operate through one or more dependent pathways in which later events may only occur if certain earlier events have occurred. This would explain the pleiotropic nature of many spo mutations. Exactly how the events of sporulation may be connected is the subject of much present research.

A major form of control over sporulation gene expression appears to be exerted at the transcriptional level while post-transcriptional controls may play a secondary role (Piggot & Coote, 1976). This is supported by the finding that new mRNAs not found in vegetative cells are synthesised during sporulation (Doi,1977) while many vegetative cell mRNAs continue to be made.

Transcriptional control

Investigations into the control of transcription in B. subtilis have concentrated on the composition and

template specificity of RNA polymerase. Although no gross changes in the core polymerase were observed during sporulation (Linn et al., 1973; Orrego et al., 1973), a number of core-associated polypeptides have been found in sporulating cells which are not present in vegetative cells. A list of the core-associated polypeptides and the forms of RNA polymerase holoenzyme found in both vegetative and sporulating cells is shown in tables 1.1 & 1.2.

Holoenzyme containing sigma factor σ^{37}, a minor vegtative cell form, was found to direct transcription of at least two cloned sporulation genes (Haldenwang & Losick, 1980; Ollington et al., 1981) which were normally turned on at the start of sporulation. Losick and Pero (1981) suggested that σ^{37}, in conjunction with spo0 regulatory gene products, could control the early stages of sporulation. At about stage II σ^{37} and some σ^{55} were replaced on RNA polymerase by σ^{29}, whose production is under spo0 control (table1.1). Each of these sigma factors discriminated between and directed transcription from promoters with distinct "-35" and "-10" sequences (Johnson et al., 1983). Holoenzyme containing σ^{29} directed transcription of further sporulation genes which may include genes for other core-associated polypeptides which could control gene expression at later stages.

Recent work (Rhaese et al., 1982) has suggested that genomic rearrangements occur during sporulation. Whether such rearrangements are a consequence of sporulation or part of its control remains to be seen.

Vegetative cells

Polypeptide	σ^{55}	δ	σ^{37}	ω1	ω2
Mr (kD)	55	21	37	11	9.5

Sporulating cells

Polypeptide	σ^{29}	p23	p34	p85
Mr (kD)	29	23	34	85

Table 1.1 RNA polymerase core-associated polypeptides of *Bacillus subtilis* (After Doi, 1982)

Vegetative cells

	composition	abbreviation
Major	$\alpha_2\beta\beta'\sigma^{55}\omega 1\omega 2$	$E\sigma^{55}$
	$\alpha_2\beta\beta'\delta\omega 1$	$E\delta$
Minor	$\alpha_2\beta\beta'\sigma^{37}$	$E\sigma^{37}$
	$\alpha_2\beta\beta'\sigma^{28}$	$E\sigma^{28}$

Sporulating cells

Developmental

Stage	Compositions Observed				
Stage 0	E	$E\sigma^{55}$	$E\sigma^{37}$	$E\delta$	
Stage II	E	$E\sigma^{55}$	$E\sigma^{29}$	EP^{34}	
Stage III	E	$E\sigma^{55}$	$E\sigma^{29}$	EP^{34}	EP^{23}

Table1.2 Compositions of RNA polymerase in vegetative and sporulating Bacillus subtilis (After Doi, 1982)

Post transcriptional control

Polyadenylated RNA with 3' polyA tracts of 120-150 nucleotides can be isolated from sporulating cells, but not from vegetative cells or cells of spo0 mutants (Szulmajster, 1982). This poly(A) RNA probably acts as mRNA and a characteristic population of poly(A) RNA is found at each stage of sporulation. Plasmids derived from poly(A) RNA by cDNA cloning methods include one which carries the spo0B gene (Kerjan et al., 1982). Whilst the function of the polyadenylation is unknown, it may offer some protection from the 3'-5' exonuclease activity found in sporulating cells, and preferentially stabilize polyadenylated species.

Translational control

Evidence for control of gene expression during sporulation by translational control has come mainly from investigations of drug-resistant mutants (Campbell & Chambliss, 1977; Smith, 1982) and analysis of components of the translational apparatus.

Selective translation of mRNA may be directed by translation initiation factor (IF) from sporulating cells. This factor showed different translational specificity from IF of either vegetative or asporogenous cells in vitro (Chambliss & Legault-Demarre, 1977).

Post-Translational control

Two main methods of control are possible at this level:

1. Modification of existing vegetative or spore proteins to form their sporulation specific counterparts.

2. Degradation of unnecessary vegetative proteins and control of protein concentration in the sporulating cell through turnover.

Protein modification, usually by limited proteolysis, is known to occur in a number of cases during sporulation. The enzyme fructose-1,6-diphosphate aldolase in vegetative cells has a molecular weight of 79kD while that of the spore aldolase is 44kD. Sadoff and coworkers (Sadoff et al., 1970) found that a 44kD aldolase with properties characteristic of the spore enzyme could be derived from the vegetative aldolase in vitro by a sporulation specific protease. This conversion also appeared to occur in vivo. A similar type of processing was found during the maturation of spore coat proteins. The major spore protein was synthesised as a 25kD precursor at about stage I, and was processed to the mature 13.5kD protein at stageII-III (Munoz et al., 1978).

Protein turnover in the sporulating cell begins at stage I and continues throughout sporulation at a rate of about 18% per hour. Turnover in vegetative cells occurs at less than 1% per hour. Although there is no difference between the rates of turnover of vegetative and sporulation proteins during sporulation, it is theoretically possible for complete turnover of all of the proteins from the vegetative cell to have occurred by the end of sporulation. This is supported by the observation that as spore proteins were synthesised, vegetative antigens were seen to decrease (Doi, 1972). Three different patterns of turnover during sporulation have been described for various enzymes (Deutscher & Kornberg, 1968) suggesting that selective

turnover of specific proteins may occur.

Control over sporulation may occur then, by many mechanisms: initiation is governed by the DNA replication cycle and the biochemistry of the stringent response; the main changes in gene expression are brought about at the transcriptional level by modifications of RNA polymerase specificity modulated by post-translational mechanisms. Further control of gene expression may occur at the translational level by template selection, while mRNA stability will determine the relative levels of various mRNA species. Protein turnover and modification probably acts as the final mechanism of control in sporulation. It produces the amino acids necessary for new protein synthesis and regulates the activity and concentration of many sporulation proteins.

1.2.4 The Cell Cycle of Caulobacter crescentus

1.2.4.1 Introduction

Caulobacter crescentus is a Gram negative freshwater bacterium which possesses an obligately dimorphic cell cycle. A simplified summary of the *C. crescentus* cell cycle is illustrated in figure 1.6. The cyclic morphological changes observed are superimposed on a DNA replication/cell division cycle similar to that of *E. coli*.

The *C. crescentus* swarmer cell is motile by virtue of its polar flagellum, but is reproductively inactive. It may become reproductively active only by differentiating to produce a stalked cell. This involves loss of the flagellum

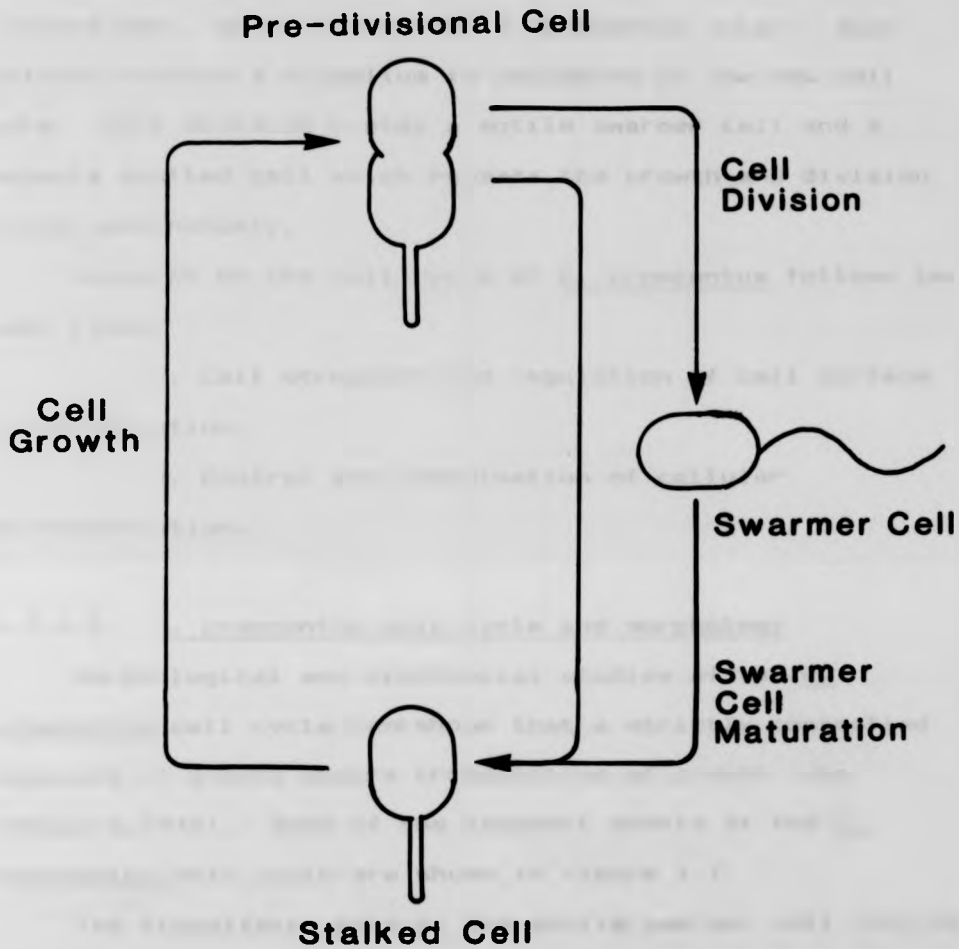


Figure 1.6 The cell cycle of *Caulobacter crescentus*.

and synthesis of a stalk or prostheca in its place. DNA replication and cell division pathways are initiated in the stalked cell, growth of which is obligately polar. Just before division a flagellum is assembled on the new cell pole. Cell division yields a motile swarmer cell and a sessile stalked cell which repeats the growth and division cycle continuously.

Research on the cell cycle of C. crescentus follows two main lines:

1. Cell structure and regulation of cell surface differentiation.

2. Control and coordination of cellular differentiation.

1.2.4.2 C. crescentus cell cycle and morphology

Morphological and biochemical studies of the C. crescentus cell cycle have shown that a strictly controlled sequence of events occurs irrespective of growth rate (Shapiro,1976). Some of the landmark events of the C. crescentus cell cycle are shown in figure 1.7.

The flagellated pole of the motile swarmer cell carries a number of other polar organelles: pili,which serve as RNA 'phage receptors, and receptors for DNA 'phage whose physical composition is unknown. All of the polar structures are lost to the medium during the maturation phase of swarmer cell differentiation (Shapiro & Maizel, 1973; Smit & Agabian, 1982), with concomitant loss of motility. Prostheca synthesis and DNA replication are then initiated simultaneously and continue throughout the rest of the cell cycle.

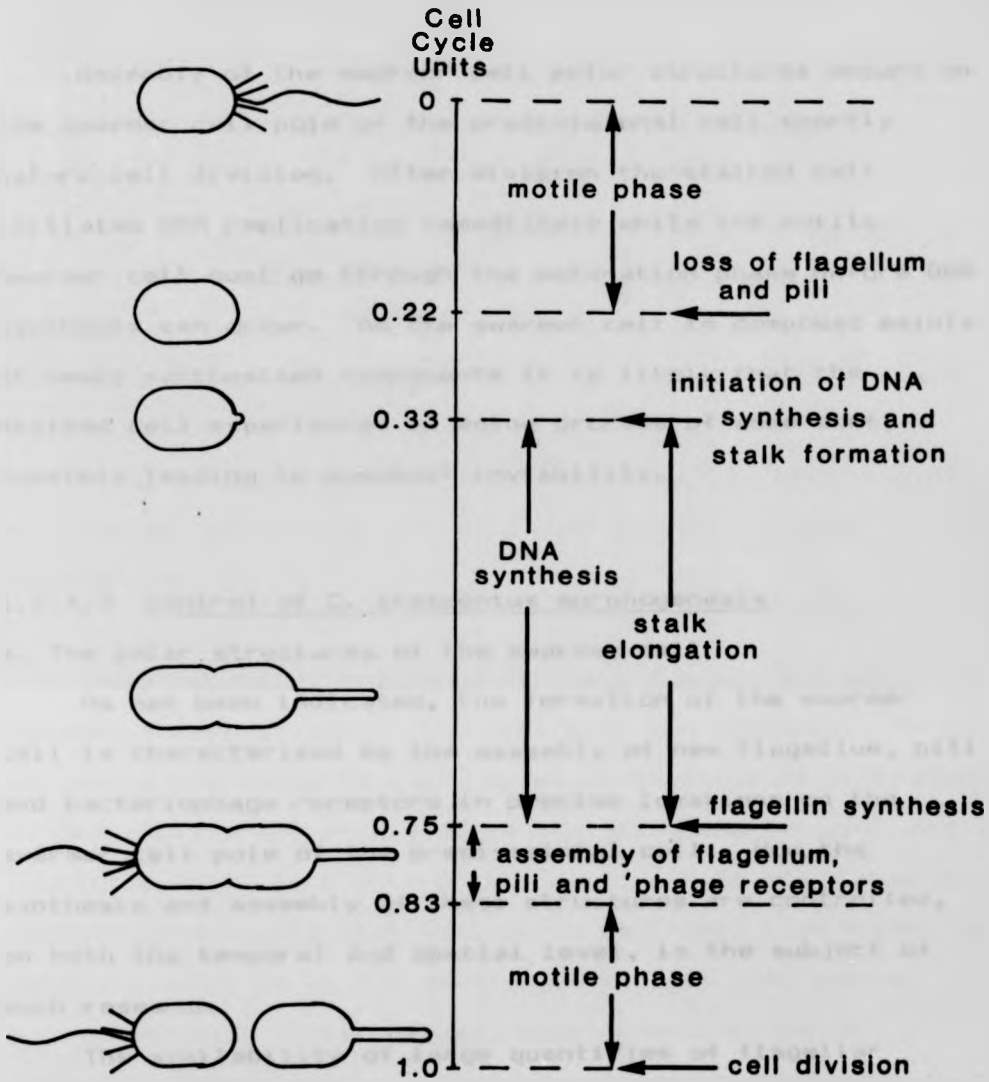


Figure 1.7 Landmark events in the C. crescentus cell cycle.

Assembly of the swarmer cell polar structures occurs on the swarmer cell pole of the predivisional cell shortly before cell division. After division the stalked cell initiates DNA replication immediately while the motile swarmer cell must go through the maturation phase before DNA synthesis can occur. As the swarmer cell is composed mainly of newly synthesised components it is likely that the stalked cell experiences an aging process of some sort, possibly leading to eventual inviability.

1.2.4.3 Control of C. crescentus morphogenesis

a. The polar structures of the swarmer cell

As has been indicated, the formation of the swarmer cell is characterised by the assembly of new flagellum, pili and bacteriophage receptors in precise locations on the swarmer cell pole of the predivisional cell. How the synthesis and assembly of these structures are controlled, on both the temporal and spatial level, is the subject of much research.

The availability of large quantities of flagellar proteins means that the production of an active flagellum is one of the most studied events of the C. crescentus cell cycle. The flagellar proteins are synthesized immediately before their assembly and this requires de novo transcription (Shapiro and Maizel, 1973). Transcription appears to be triggered by an event late in the DNA replication cycle (Osley et al., 1977). Flagellin proteins are found only within the swarmer cell after division due to the segregation of flagellin mRNA with the swarmer cell (Agabian et al., 1979; Milhausen and Agabian 1983), but the

mechanism by which this is acheived has yet to be discovered. Two mechanistic possibilities have been suggested:

1. Differential transcription from the swarmer and stalked cell nucleoids within the predivisional cell. The nucleoids are known to have characteristically different configurations, even within the predivisional cell (Evinger & Agabian, 1979).

2. Compartmentalisation of flagellin mRNA within the swarmer cell, possibly by a membrane or ribosome interaction.

More recently, isolation of the pilus protein has allowed investigation of its synthesis and assembly. Pilin synthesis was found to begin at about the time of initiation of DNA replication in the differentiating swarmer cell and continued until late in the cell cycle. Pili are assembled shortly before cell division, so it is likely that a pool of pilin is built up in the predivisional cell long before pilus assembly. This suggests that some stimulus or function needed for pilus assembly appears at about the time of flagellum assembly (Smit & Agabian, 1982).

It is possible that some features of the assembly apparatus are shared as mutants can be isolated in which both flagellum and pili are missing and DNA 'phage receptors are altered. Revertants regain all of the wild type properties simultaneously, indicating that a single mutation is responsible for all of the effects observed (Shapiro, 1976).

b. Stalk Morphogenesis

As stalk growth occurs only at the cell/stalk junction, stalk synthesis in the developing C. crescentus cell consists of localised synthesis of cytoplasmic membrane, mucopeptide, lipopolysaccharide and outer membrane proteins. It is under cell cycle control and will not occur until after cell division. This implies that stalk formation is dependent on some previous cell division step (Shapiro, 1976).

1.2.4.4 Control of the cell cycle

The timing of landmark events in the C. crescentus cell cycle is strictly controlled ensuring that they occur at the same point in the cell cycle irrespective of growth rate (see figure 1.7). This timing appears to be determined by the DNA replication pathway. DNA replication is initiated simultaneously with prostheca formation in the differentiating swarmer cell, and immediately after cell division in the stalked cell. The length of the DNA replication phase varies with growth rate so that cell cycle events are kept in step with growth rate.

Evidence that DNA replication does control the timing of cell cycle events came first with the discovery of "DNA execution times" for various cell cycle events. Occurrence of these events is dependent on replication of a specific amount or region of the chromosome. Examples, with their DNA execution points in cell cycle units are:

1. Flagellar hook protein synthesis; 0.61
2. Flagellin synthesis; 0.65
3. Initiation of cell division; 0.74

4. Cell separation; 0.85

(Sheffery & Newton, 1981)

The cell cycle of C. crescentus, like that of B. subtilis, appears to be regulated by a dependent sequence of events. In C. crescentus, however, the central event in the pathway appears to be DNA replication. By blocking cell cycle events at various stages it was found that the main dependent pathway in C. crescentus linked DNA replication, cell division, and swarmer cell differentiation. DNA replication is required for the initiation of cell division and cell division is required for subsequent swarmer cell maturation and stalk formation. A number of other events were found to be a consequence of the main pathway but were not required for later events. These included formation of the swarmer cell polar structures on the predivisional cell (Shapiro, 1976). Further investigation revealed that DNA replication and cell division formed two distinct but linked pathways. Initiation of the cell division pathway is dependent on a late stage of DNA replication . The cell division pathway proceeds to produce a septated predivisional cell but cell separation does not occur without prior termination of DNA replication (Osley & newton, 1980).

Nathan et. al. (1982) have shown that initiation of DNA replication is dependent on a late stage of the previous DNA replication pathway, possibly termination. This indicates that dichotomous replication is not likely to occur in C. crescentus and that the DNA replication pathway is circular in nature.

The mechanism by which DNA replication controls gene

expression is unknown, but appears to act at the level of transcription (Newton, 1972). How transcriptional control is exerted also remains unclear. It does not, however, appear to be due to alterations of the composition of the RNA polymerase (Amemiya et al., 1977). A promising possibility is that the configuration of the nucleoid acts to regulate transcription, possibly through alterations in local DNA tertiary structure. It is known that stalked and swarmer cells, which have different transcriptional abilities, have nucleoids with different conformations (Evinger & Agabian, 1979).

It has also been found that a DNA rearrangement occurs during the cell cycle, associated with inverted repeat sequences (Nisen et al., 1979). Such an event might be involved in the activation of specific cell cycle loci. This possibility will be discussed further in section 1.3.

1.2.5 Morphogenesis and Differentiation in Rhodomicrobium vannielii

1.2.5.1 Introduction

Rhodomicrobium vannielii (Duchow & Douglas, 1949) is a member of the Rhodospirillaceae which possesses a polymorphic cell cycle. It is Gram negative and reproduces by an obligately polar mechanism. In liquid batch culture R. vannielii appears to exist as small, ovoid cells 2-3 microns long connected by filaments which may be branched, forming ramifying multicellular arrays. Single, motile swarmer cells (Douglas & Wolfe, 1959) and non-motile cells

carrying polar filaments may also be seen. Under adverse conditions pyramidal, heat resistant exospores are also found (Whittenbury & Dow, 1977). The filament or prostheca of R. vannielii, unlike that of C. crescentus, is obligately used in reproduction, daughter cells being formed at the tip of the prostheca. Cell separation in R. vannielii occurs by deposition of a cross wall within the prostheca, except in the generation of swarmer cells or exospores where asymmetric cell division occurs at the end of the prostheca.

1.2.5.2 The cell cycle of Rhodomicrobium vannielii

Rhodomicrobium vannielii has a polymorphic cell cycle in which either of two vegetative cycles may be followed and at least three distinct cell morphologies can be observed.

Swarmer cells of R. vannielii are produced under conditions of low light and high CO_2 tension. These cells are reproductively inactive and have reduced metabolic activity; there is no DNA or rRNA synthesis, and protein synthesis is at a reduced rate (Dow et al., 1983). The swarmer cell has several flagella which are arranged peritrichously, but unlike the C. crescentus swarmer cell it has no obvious polar structures. Upon an increase in light intensity under favourable nutrient conditions, R. vannielii swarmer cells go through an obligate morphogenetic sequence which culminates in cell division. This response to growth conditions has led to the suggestion that swarmer cells of R. vannielii form a dispersal phase produced under low nutrient conditions (Dow & Lawrence, 1980; Dow et al., 1983).

Differentiation of R. vannielii swarmer cells can be divided into two stages; maturation and reproduction (Whittenbury & Dow, 1977). During the maturation phase rRNA synthesis begins and the swarmer cell becomes non-motile by shedding its flagella. The reproductive phase is characterized by the synthesis of a prostheca at one or both cell poles and the initiation of DNA replication. The distal tip of the prostheca then swells to form a daughter cell bud which grows to about the same size as the mother cell. The final stage of differentiation, daughter cell completion, involves physiological separation from the mother cell. This may be by cross wall formation or binary fission at the prostheca/daughter cell junction depending on the type of cell formed. The landmark events of R. vannielii swarmer cell differentiation are shown in figure 1.8.

The same mode of reproduction is used by cells within a multicellular array. Filament formation may be from either pole, or by branching of an existing filament on the cell-proximal side of a cross wall. Each cell may produce a maximum of four daughter cells (Whittenbury & Dow, 1977), suggesting that R. vannielii cells exhibit mortality and have a strictly limited reproductive potential. This may be an extension of the aging process exhibited by C. crescentus stalked cells.

When grown under conditions of high light and low CO_2 tension the "complex" cell cycle is followed, producing characteristic branching multicellular arrays. If the light intensity is reduced and the CO_2 tension increased, as normally occurs in the late exponential phase of batch

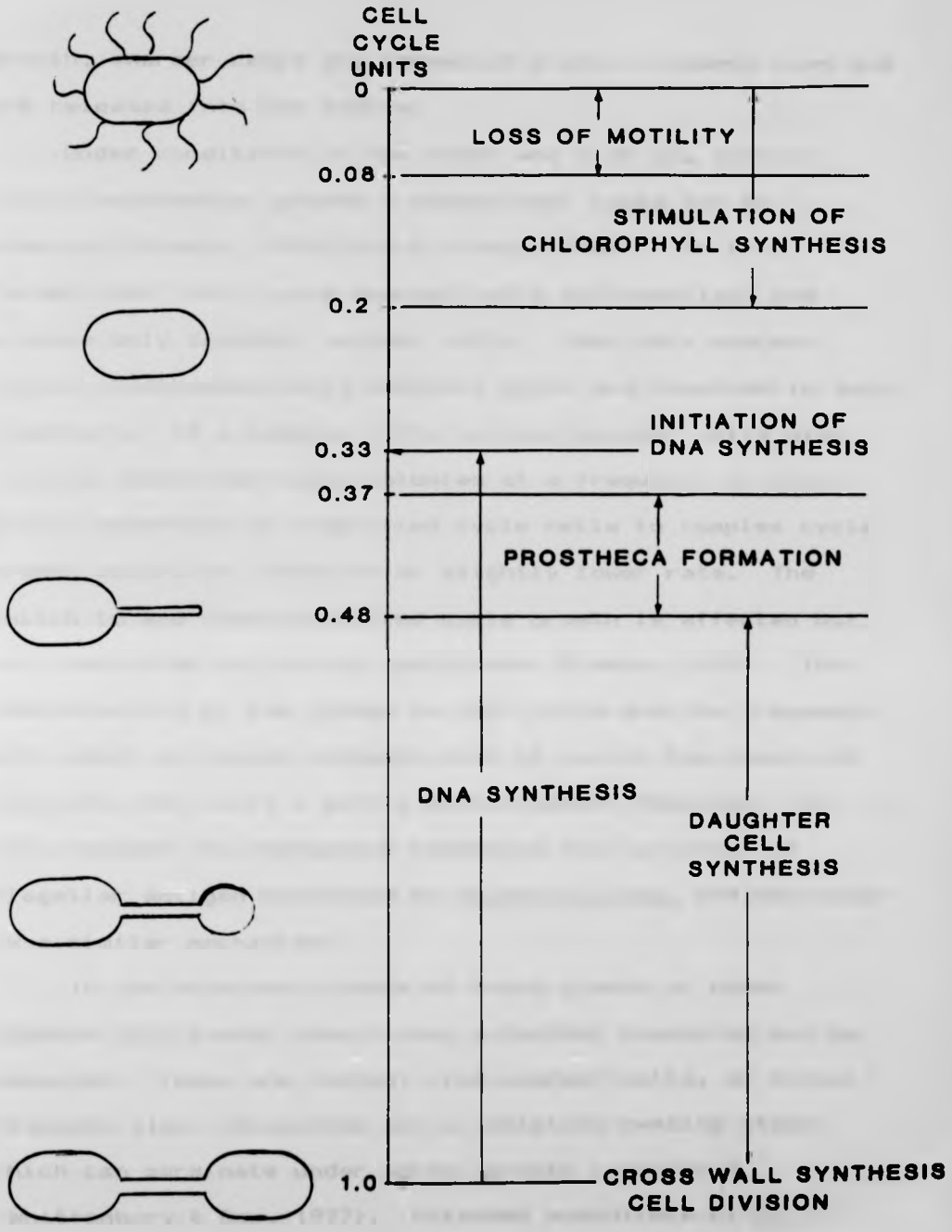


Figure 1.8 Landmark events in *R. vannielii* swarmer cell differentiation.

growth, swarmer cells are formed at distal filament tips and are released into the medium.

Under conditions of low light and high CO_2 tension during exponential growth a second cell cycle may be observed (France, 1978; Dow & France, 1980). In this "simplified" cell cycle swarmer cells differentiate and produce only daughter swarmer cells. Thus only swarmer cells, prosthecate cells and cell pairs are observed in such a culture. In a complex cycle culture swarmer cells give rise to simplified cycle colonies at a frequency of about 0.1%. Reversion of simplified cycle cells to complex cycle growth occurs at a similar or slightly lower rate. The switch to and from simplified cycle growth is affected but not controlled by culture conditions (France, 1978). The reversibility of the change in cell cycle and the frequency with which it occurs suggests that it is not the result of mutation, nor is it a purely physiological response. In this respect the phenomenon resembles the variation of flagellar antigen exhibited by Salmonella spp. and may occur by a similar mechanism.

In the stationary phase of batch growth or under adverse nutritional conditions, pyramidal exospores may be observed. These are formed, like swarmer cells, at distal filament tips. Exospores are a resistant resting stage which can germinate under normal growth conditions (Whittenbury & Dow, 1977). Extended subculture of R. vannielii under laboratory conditions however, leads to loss of the exospore phenotype. This does not appear to be due to a simple mutation as exospores can be observed at low frequencies in later cultures, but resembles the change from

complex to simplified cell cycle (C.S. Dow, personal communication). The complete cell cycle of R. vannielii is shown diagramatically in figure 1.9.

Thus R. vannielii cells appear to follow three rules to determine the nature of daughter cells:

1. In good growth conditions (high light, low CO_2) form reproductive cells.
2. In moderate growth conditions (low light, high CO_2), form swarmer cells.
3. In poor growth conditions (nutrients exhausted), form exospores.

1.2.5.3 Control of the cell cycle

Research on R. vannielii is concerned mainly with the differentiation of the swarmer cell. The characteristic morphological changes which occur during differentiation allow precise determination of the developmental stage to be made by microscopic or cell volume analysis.
Differentiation takes approximately 6 hours under laboratory conditions and the variations in morphology observed with time are shown in figure 1.10. Populations of inhibited R. vannielii swarmer cells, selected by filtration in darkness, can be induced to differentiate synchronously by re-illumination. This allows investigation of the biochemical events which accompany the morphological changes observed and is one of the features which makes R. vannielii an attractive subject for the investigation of bacterial differentiation.

In the inhibited swarmer cell DNA synthesis is absent and RNA and protein synthesis occur at reduced rates. At

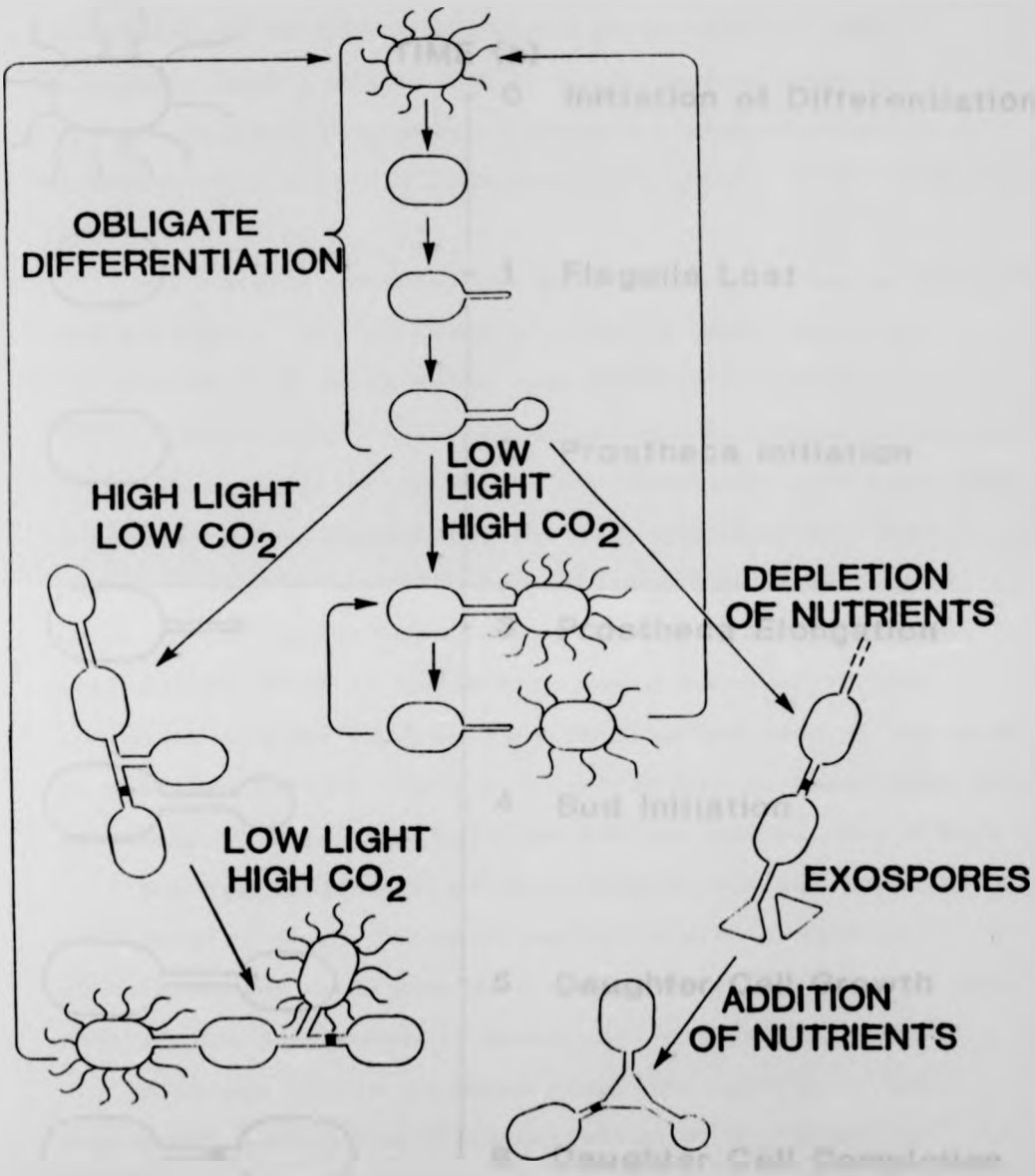


Figure 1.9 The polymorphic cell cycle of R. vannielii.

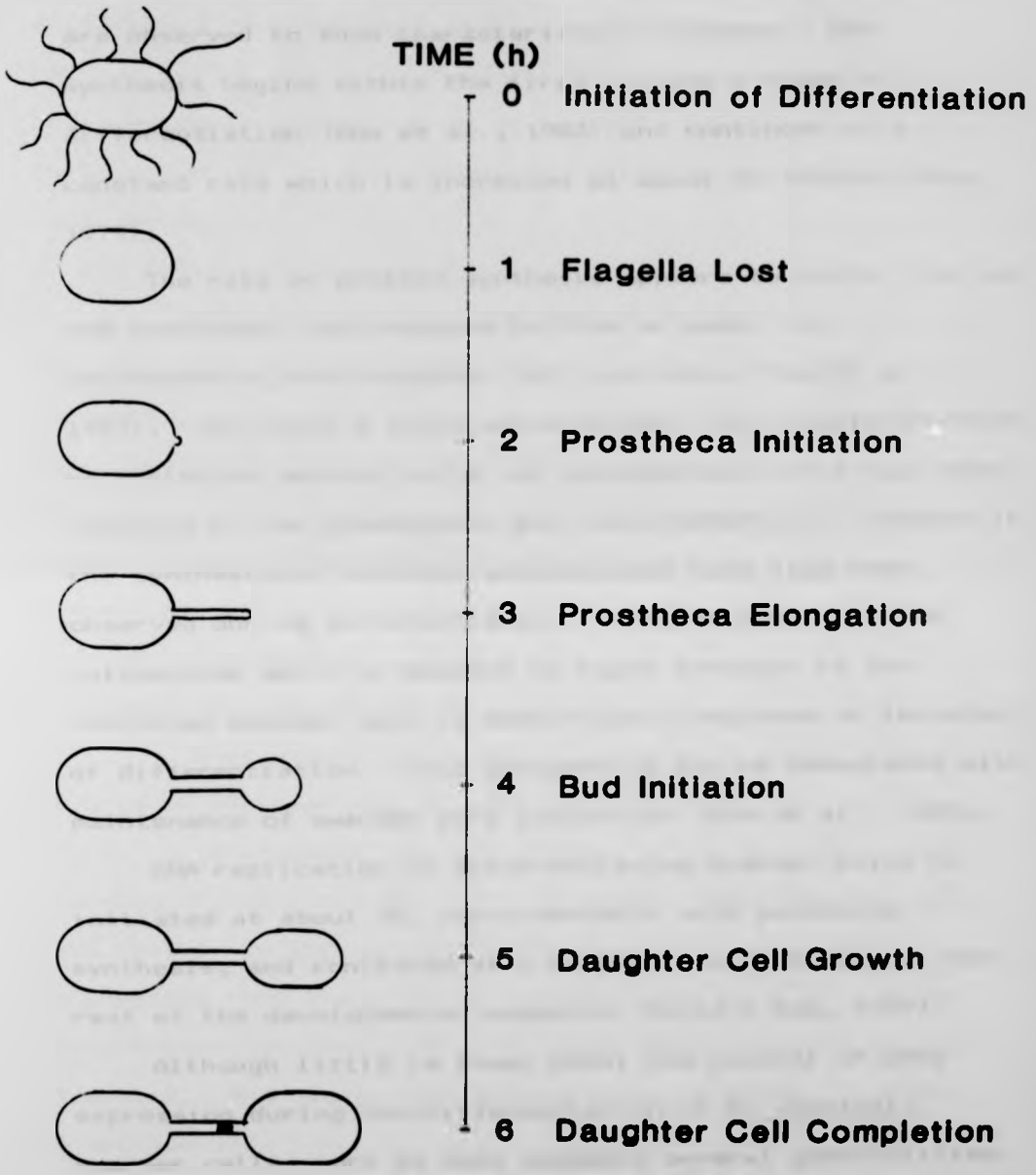


Figure 1.10 Morphological changes observed during differentiation of *R. vannielii* swarmer cells.

the onset of differentiation however, all three processes are observed to show characteristic increases. RNA synthesis begins within the first fifteen minutes of differentiation (Dow et al., 1983) and continues at a constant rate which is increased at about 3h (Potts & Dow, 1979).

The rate of protein synthesis appears to mirror that of RNA synthesis, the increase in rate at about 3h corresponding with daughter cell synthesis (Dow et al., 1983). Qualitative differences between the soluble proteins of inhibited swarmer cells and reproductive cells have been observed by two dimensional gel electrophoresis. Changes in the synthesis of specific polypeptides have also been observed during differentiation. Interestingly, a 12kD polypeptide which is subject to rapid turnover in the inhibited swarmer cell is specifically degraded at the start of differentiation. This polypeptide may be associated with maintenance of swarmer cell inhibition (Dow et al., 1983).

DNA replication in differentiating swarmer cells is initiated at about 2h, coincidentally with prostheca synthesis, and continues at a constant rate throughout the rest of the developmental sequence (Potts & Dow, 1979).

Although little is known about the control of gene expression during the differentiation of R. vannielii swarmer cells, work to date suggests several possibilities.

1. Nucleoid conformation

The nucleoid of the swarmer cell, which is compact and centrally located, undergoes a change in conformation after initiation of differentiation to become elongated and moves toward the pole at which the prostheca will form

(Whittenbury & Dow, 1977). This position is maintained until after daughter cell completion when the nucleoid returns to the centre of the cell but retains its extended conformation. These changes in nucleoid conformation may be involved in the transcription and replication of the swarmer cell genome.

2. RNA polymerase modification

Recent work has shown that although no qualitative changes can be found, quantitative changes in RNA polymerase core-associated polypeptides occur during differentiation. Specifically, a decrease in a 95kD polypeptide and an increase in a 70kD polypeptide are observed (Dow et al., 1983). These changes may bring about a switch in transcriptional specificity which stimulates expression of developmental genes.

3. Genome rearrangements

Rhodomicrobium vannielii possesses unusually large amounts of inverted repeat sequence DNA (Potts et.al., 1980). Such sequences are ubiquitous in translocatable DNA elements (Calos & Miller, 1980; Kleckner, 1981) and may also have effects on DNA secondary structure (Lilley, 1980). It is possible that the unidirectional switch by which swarmer cells become reproductively active involves a rearrangement of specific DNA sequences. This will be considered further in section 1.3.

1.3 Inverted Repeat Sequences

1.3.1 Introduction

In this section a few of the structural and functional properties of inverted repeat sequences will be discussed. Interest in this type of sequence stems from the ability of each of the strands of any inverted repeat sequence to anneal with itself to form a duplex region of DNA.

i.e.

Inverted repeat sequences possess 180° rotational symmetry, sometimes called twofold or dyad symmetry, about a point midway between the repeats. The non-repeated DNA between the repeats may be of any length and where the intervening gap is small (<10bp) the inverted repeat structure may be called a palindrome.

These sequences were originally observed as a very rapidly renaturing fraction of DNA from both prokaryotes and eukaryotes (Bernardi, 1962; Alberts & Doty, 1968; Walker & McLaren, 1969). This concentration- independent renaturation was initially thought to be due to covalent cross-links between the strands of duplex DNA (Alberts & Doty, 1968), but was later shown to be due to the presence of inverted repeat sequences (Wilson & Thomas, 1974).

These sequences appear to be a ubiquitous feature of all DNAs and in some cases are present in quantities greater than predicted by statistical methods (Day & Blake, 1982). This has led to the suggestion that inverted repeat

sequences have some function or property which has allowed their conservation in evolution. Inverted repeat sequences are required in most DNA rearrangements and are involved in some mechanisms of control of gene expression. They are present at the ends of transposable and invertible elements; act as sites for protein recognition, initiation of DNA replication, termination of trancription and processing of RNA molecules; and may give rise to unusual DNA secondary structures. The presence of this class of sequences in bacteria which exhibit differentiation, in quantities similar to that found in eukaryotes, suggests that the investigation of the function of inverted repeat sequences in differentiating bacteria might yield valuable results. This will be discussed further in section 1.3.4.

1.3.2 Distribution and Abundance of Inverted Repeat Sequences

1.3.2.1 Eukaryotic inverted repeats

Inverted repeat sequences appear to be a general feature of eukaryotic DNA. They have been found in the DNA of many organisms, their organelles, and in viruses. Wilson and Thomas (1974) determined that for *Drosophila spp.*, mouse and human DNA at least, inverted repeats average 300-1200bp in length and are arranged in small clusters interspersed throughout the genome. This distribution was confirmed by Schmid et al. (1975) who found inverted repeat sequences of up to 15kb interspersed every 40-80kb in *Drosophila* DNA. They estimated that a total of 2000-4000 inverted repeat pairs are present in the *Drosophila* genome, accounting for

about 3% of the total DNA.

1.3.2.2 Prokaryotic inverted repeats

The DNA of most prokaryotes contains much lower amounts of all classes of repeated sequences than are found in eukaryotes. E. coli, originally reported to contain no inverted repeat sequences in its DNA (Wilson & Thomas, 1974), was found by enrichment of repetitive sequences to have 0.5% inverted repeat sequence, and a further 2% repetitive fraction in its genome (Kato et al., 1974). Inverted repeat sequences from E. coli have since been visualised by electron microscopy and analysed by agarose gel electrophoresis (Chow, 1977; Ohtsubo & Ohtsubo, 1977). Duplex fragments corresponding in size to insertion sequence (IS) elements, and others of around 1kb were found. Inverted repeat sequences can also be found in the DNA of plasmids and 'phages of E. coli and in the genomes of other Enterobacteriaceae (Ohtsubo & Ohtsubo, 1976; 1977). It now appears that inverted repeat sequences may be found in the genomes of most prokaryotes and form four classes.

1. Large inverted repeats (>2kb) corresponding to duplicated genes or operons (Hill & Harnish, 1981).
2. Moderate inverted repeats (0.1-2kb) including repeated IS-like elements (Ohtsubo & Ohtsubo, 1976; 1977; Chow, 1977).
3. Short inverted repeats (20-50bp) including sequences such as those found at the ends of transposable or invertible elements (Calos & Miller, 1980; Hsu & Davidson, 1974).
4. Very short inverted repeats/palindromes (<20bp) including sites for sequence-specific protein binding (Modrich, 1982; Gicquel-Sanzey & Cossart,1982); termination of transcription

(Holmes et al., 1983) and RNAse III cleavage (Gottesman et al., 1982).

There have been a few reports of bacteria possessing larger amounts of inverted repeat sequence DNA. Specifically, Bacillus subtilis (Galloway & Rudner, 1979), Caulobacter crescentus (Wood et al., 1976) and Rhodomicrobium vanneilii (Potts et al., 1980) have all been found to have about 5% of their DNA as inverted repeat sequences. The role of the inverted repeat DNA in these bacteria is unknown but it is tempting to speculate that it might play some role in the cellular differentiation exhibited by these three species.

1.3.3 Structural and functional aspects of inverted repeat sequences

As has already been mentioned, inverted repeat sequences have been found to be involved in several genetic processes. In this section some of these processes will be described and the role played in each case by inverted repeat sequences will be discussed. Wherever possible, examples from both prokaryotic and eukaryotic systems will be used.

1.3.3.1 Transposition of DNA segments

In prokaryotes, almost all of the transposable elements studied have inverted terminal repetitions. These repeats range in size from a few base pairs for the IS elements and the Tn3 family to over 1kb in the case of composite elements like Tn5 and Tn10 (Kleckner, 1981). Prokaryotic

transposable elements have been divided into three groups on the basis of their structure and properties (Kleckner, 1981).

IA. Insertion sequence (IS) -like elements. These are short 0.8-1.5kb elements with inverted terminal repeats of up to 50bp. They may encode their own tranposition proteins but are otherwise phenotypically silent.

IB. Composite elements. These are made up of phenotypic determinants (usually drug resistance genes) flanked by direct or inverted repeats of an IS-like element.

II. The Tn3 family. Members of this family of transposable elements are at least 5kb long with inverted repeat ends of 35-38bp which show considerable homology between different elements.

Note that the transposition of class I and II elements appears to differ both in its regulation and mechanism (Reznikoff, 1982; Grindley, 1983).

III. Transposable bacteriophages. Bacteriophage Mu replicates wholly by transposition mechanisms (Toussaint et al., 1977). As no inverted repeat sequences appear to be involved in this tranposition it will not be discussed further.

The involvement of inverted repeat sequences in transposition appears to be in the provision of symmetrically arranged sites for the binding of specific transposition proteins at the transposon ends. In the class I transposon Tn10, all of the sites required for transposition are found within the first 25bp of each end. These appear to be the sites of action of the Tn10 transposase (Foster et al., 1981a). Similar involvement of

the inverted repeats seems to occur in the other class I elements including Tn5 (Reznikoff, 1982). The situation for class II transposons is less clear. Although the ends are required for transposition, no interaction with the transposase protein has yet been demonstrated for Tn3 (Grindley, 1983).

Precise and nearly-precise excision of class I transposons also appears to involve the inverted repeat sequences. In these events the inverted repeats may act by bringing together the ends of the transposon to allow recombination to occur between them by some specialised mechanism unrelated to transposition (Foster et al., 1981b; Egner & Berg, 1981).

The archaebacteria also contain transposable elements with terminal inverted repeats. Halobacterium halobium contains a series of elements ranging in size from 750bp to 3kb with 10bp terminal inverted repeats (Pfeifer et al., 1983). Such elements may be responsible for the high frequency of DNA rearrangement observed in Halobacterium spp. (Sapienza & Doolittle, 1982; Sapienza et al., 1982).

Transposable elements in eukaryotes exhibit a variety of structures, some of which are similar to those of prokaryotic elements. Three classes of eukaryotic transposable elements have terminal inverted repeat sequences. These are:

1. Elements with long direct terminal repeats.

These elements, including the COPIA elements of Drosophila spp. and Ty1 from yeast, make up part of the middle repetitive DNA of eukaryotic genomes and have

structures similar to that of the prokaryotic composite transposons. Typically they have terminal direct repeats of 250-500bp, each of which is flanked by inverted repeats of the order of 10bp long. Note that although the flanking repeats of some prokaryotic composite transposons are capable of independent transposition, their eukaryotic counterparts are not.

2. Elements with long terminal inverted repeats

The best known of these elements are the foldback (FB) transposable elements of Drosophila melanogaster. The members of this family of transposable elements are heterogenous both in total size and in size of the inverted repeat regions, but appear to share homology within the inverted repeats (Potter et al., 1980). These are made up of repeated blocks of sequence 10-30bp long and the ends of the inverted repeats are highly conserved among different FB elements (Truett et al., 1981). The inverted repeat sequences of FB elements have also been implicated in a deletion event (Potter, 1982), and in precise excision of the element (Collins & Rubin, 1983).

3. Elements with short terminal inverted repeats

One form of hybrid dysgenesis in Drosophila melanogaster is due to the mutational effects associated with a class of transposable elements called the P elements. The prototype element of this family is 2.9kb long with 31bp terminal inverted repeats. Derivatives of this element associated with the mutational effects of hybrid dysgenesis carry deletions of internal portions of the element but not of the ends, suggesting that the ends of these elements are important in transposition (O'Hare & Rubin, 1983).

1.3.3.2 DNA Inversions

Homologous recombination between inverted repeat sequences will lead to the inversion of any sequences found between them, as is illustrated in figure 1.11. The consequences of such an event will depend largely on the length and nature of the sequence which is inverted. Several possibilities exist.

1. Inversion of regulatory sequences, which may lead to changes in transcription.

2. Inversion of protein coding sequences, which may lead to the production of truncated or otherwise altered polypeptides.

3. Inversion of whole genes or operons, which may have little or no obvious effect on gene expression.

An example of the first type of inversion event is found in the regulation of flagellar phase variation in *Salmonella spp.*. The composition of the *Salmonella typhimurium* flagellum is determined by an invertible region of DNA which carries the promoter for the adjacent H2 flagellar antigen gene but none of its protein-coding sequence (Simon et al., 1980). In one orientation expression of the H2 gene is allowed and a downstream gene (*rH1*) is also expressed. The *rH1* gene product is a repressor of the unlinked H1 flagellar antigen gene and so only H2 expression occurs. When the orientation of the invertible segment is reversed, neither the H2 nor the *rH1* genes can be expressed and so expression of the H1 gene is allowed. The invertible region consists of a 970bp segment of DNA flanked by 14bp inverted repeat sequences which are

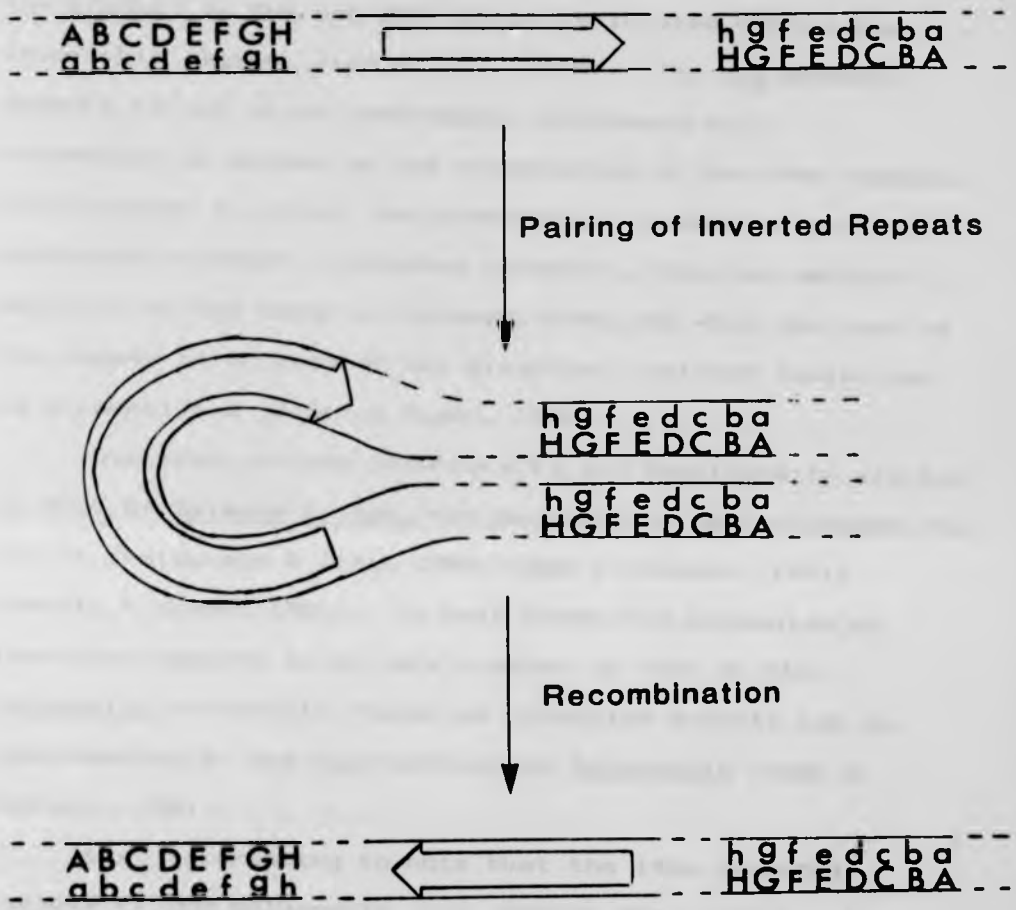


Figure 1.11 DNA inversion by recombination between inverted repeat sequences.

absolutely required for inversion. Inversion of the controlling element is recA independent and is catalysed by the product of the hin gene which is located within the invertible region (Zieg et al., 1978). The hin protein appears to act as an homologous, site-specific, recombination enzyme as the orientation of the 14bp repeats can be shown to effect the products of recombination in a predictable manner. Repeated directly, they can mediate deletion of the material between them; and when one copy of the repeat is on each of two plasmids, replicon fusion can be accomplished (Scott & Simon, 1982).

Inversion systems structurally and functionally similar to that of Salmonella spp. can be found in bacteriophages Mu and P1 (Kutsukake & Iino, 1980; Kamp & Kahmann, 1981; Szekely & Simon, 1981). In both cases the mechanism of inversion appears to be very similar to that of the Salmonella invertible region as inversion mutants can be complemented by the hin function of Salmonella (Kamp & Kahmann, 1981).

It is interesting to note that the 14bp inverted repeats of the Salmonella hin region show good homology with the outermost ends of the inverted repeats surrounding the invertible regions of both Mu and P1. This homology, coupled with the functional similarities between the inversion enzymes suggests that the ends of the inverted repeats are the specific sites of action of the inversion enzymes and as such will be absolutely required for inversion.

Larger inverted repeat regions may also give rise to inversions by homologous recombination. Recombination

between the ribosomal RNA operons rrnB or E and rrnD leads to the inversion of approximately 20% of the E. coli chromosome, a segment including the origin of replication. This inversion apparently has little effect on growth and may occur at a frequency of 1% (Hill & Harnish, 1981).

Among eukaryotes, the circular DNA of plant chloroplasts contains inverted repeats of 10-25kb, including the ribosomal 16s and 23s RNA genes, separated by about 20kb on one side and 80kb on the other. Palmer (1983) has reported that in the bean at least, chloroplast DNA exists as equimolar populations carrying the two orientations of the unique sequence DNA. It is likely that this is a result of inversion involving the inverted repeat sequences, although this has not yet been shown.

The 2 micron plasmid of yeast is a cirle of 6318bp containing inverted repeats of 599bp which divide the plasmid into two equally sized segments. Recombination can occur between the inverted repeats, leading to changes in the relative orientation of the unique regions. Recombination occurs at a specific site in the inverted repeats, localised within a 65bp fragment which itself contains regions of dyad symmetry, and is catalysed by the 2 micron-coded protein FLP which appears to be the sole protein requirement for recombination. Inversion may also occur by homologous recombination between the inverted repeat sequences at a much lower frequency (Broach, 1982; Broach et al., 1982; Cox, 1983).

The genome of the double-stranded DNA virus Herpes Simplex (HSV) has an unusual sequence structure which is illustrated in figure 1.12a. Virion DNA is composed of two

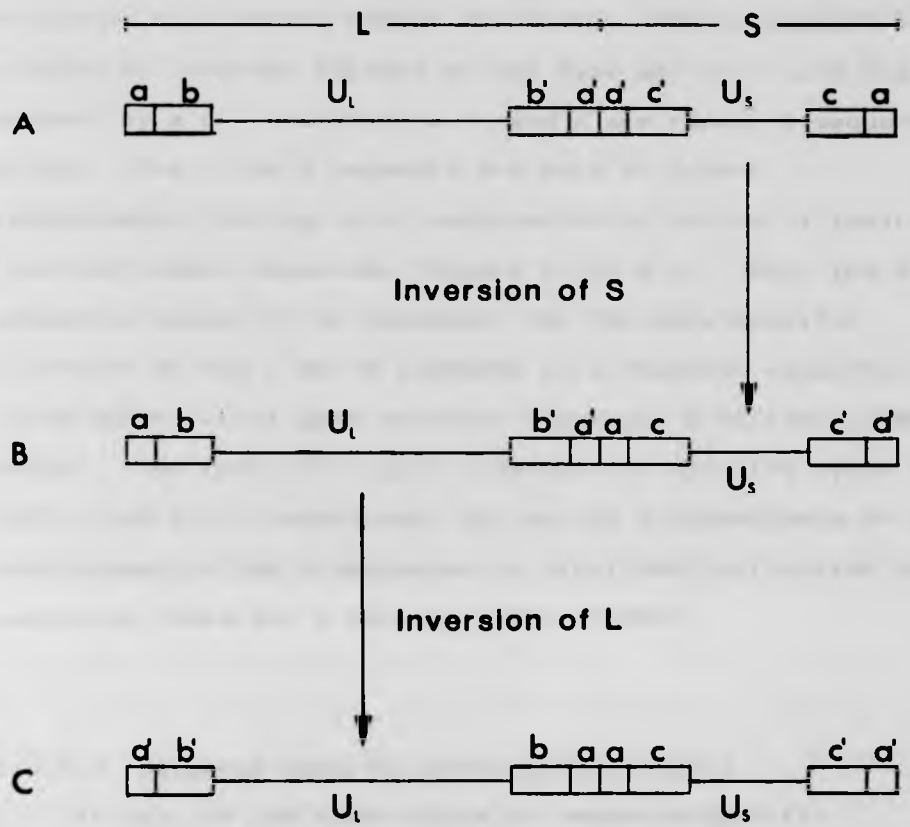


Figure 1.12 Sequence structure and inversion of Herpes Simplex virus DNA.

unique components termed U_L and U_S, each of which is bracketed by inverted repeat sequences. The U_L segment is flanked by inverted repeats of the type ab---b'a' and the U_S segment by a'c'---ca where a, b and c are repeated sequence blocks. The L and S segments are able to invert independently during viral replication by virtue of their inverted repeat sequences (figure 1.12b & c). Only the a sequences appear to be necessary for the site-specific inversion of the L and S segments in a reaction requiring trans-acting viral gene products (Mocarski & Roizman, 1981; 1982a). The role of L and S inversion in the life cycle of HSV is not fully understood, but may be a consequence of the involvement of the a sequences in viral DNA replication and packaging (Mocarski & Roizman, 1981; 1982b).

1.3.3.3 Sequence-specific DNA-protein binding

In many of the known cases of sequence-specific DNA-protein binding, both the protein and the DNA sequence to which it binds have twofold symmetry (Modrich, 1982; Gicquel-Sanzey & Cossart, 1982). The possibility that inverted repeat sequences could form unusual secondary structures (Wilson & Thomas, 1974) leads to the suggestion that such structural features might be important in protein binding. An alternative explanation is that the protein recognises the DNA sequence directly in the double-stranded molecule. It is also possible that a combination of structure and sequence recognition occurs.

Recognition of specific sequences in double-stranded DNA must necessarily involve interactions between the bases

of the DNA molecule and the amino acid side chains of the protein binding site. Mechanisms by which such interactions might occur in the major or minor groove of duplex DNA have been suggested (Seeman et al., 1976; Church et al., 1977). Investigation of well characterised DNA-binding proteins and their interaction with DNA has yielded valuable results.

The cro protein of phage λ was found to bind as a dimer at a 17bp sequence with almost perfect dyad symmetry. Elucidation of the 3-dimensional structure of cro protein suggested that binding was mediated by a pair of parallel alpha-helices which appeared to bind in the major grooves of the operator DNA (Anderson et al., 1981), sequence specificity being determined by hydrogen bonding between free amino acid side chains and the exposed areas of the DNA base pairs (Ohlendorf et al., 1982).

Comparison of the amino acid sequences of several sequence-specific DNA-binding proteins showed considerable homology. This homology extended mainly over an area including the DNA binding helices of cro protein and suggested that this group of proteins shared a mechanism of DNA binding and were probably evolutionarily related (Anderson et al., 1982; Matthews et al., 1982; Gicquel-Sanzey & Cossart, 1982). These proteins included repressors from E. coli, its phages, and phages of Salmonella spp.. Homology also existed between the DNA sequences bound by these proteins; most of them containing a core consensus sequence with variations in the sequences around the core (Gicquel-Sanzey & Cossart, 1982).

These proteins appear to form a class of evolutionarily related DNA-binding proteins, with similar modes of DNA

binding and sites of action. They may not however, represent the only mechanism of sequence-specificity but may represent that used in the majority of cases where symmetrical binding sites are involved.

Short palindromic sequences, then appear to be a favoured sequence structure for at least one class of sequence-specific DNA-binding proteins. This may be due to the relative simplicity with which specificity can be increased by symmetrical duplication of both protein and DNA binding sites during evolution.

1.3.3.4 Secondary structural properties of inverted repeat sequences

Even before inverted repeats were demonstrated as features of DNA sequences the possibility of their existence as alternative secondary structural features had been recognised (Platt, 1955). It was thought possible that such cruciform structures might exist in equilibrium with the normal DNA structure and provide physical markers on DNA molecules (Wilson & Thomas, 1974). Whether cruciform DNA actually exists *in vivo* is a question which still remains unresolved although much work has been done towards defining the effects of palindromic sequences on DNA structure.

In vitro experiments have shown that in supercoiled plasmid molecules even very short palindromes formed highly specific sites for single-strand specific nucleases or DNA modifying agents (Lilley, 1980; 1983; Panayotatos & Wells,1981). The explanation advanced to account for these results was that an alternative, probably cruciform,

conformation had been adopted by the inverted repeat regions to relieve the torsional strain imposed by supercoiling, and that the single-stranded loops of these structures were the sites recognised by the single-strand specific agents. That supercoiling increased the probability of cruciform formation was confirmed by work using topoisomers of palindrome-containing plasmids (Panyutin et al., 1982; Mizuuchi et al., 1982). Kinetic studies on a 13bp-palindrome containing plasmid suggested that cruciform formation occurred by gradual extrusion rather than by a one-step isomerisation process (Lilley & Markham, 1983).

Other work however (Courey & Wang, 1983; Sinden et al., 1983), suggested that cruciform DNA was not found *in vivo* but was formed during plasmid extraction. These workers proposed that although the degree of supercoiling found *in vivo* was sufficient to support the formation of cruciforms, they were not actually formed because the initial steps of crucifom extrusion could not occur. This may have been because:

1. The torsional strain of supercoiling was taken up in topologically constrained domains.
2. Topoisomerase activity has removed the torsional strain present in the DNA.
3. The energy required for the initial unpairing of the loop section of the palindromic sequence was not available.

This suggests that if cruciform structures are formed *in vivo* they may be the result of some selective process by allowing superhelical torsion or by the presence of helix-destabilising agents in regions containing inverted repeat sequence DNA.

The value, then, of inverted repeat sequences as secondary structural markers on DNA may depend on other intracellular factors. These factors probably include the extent of local supercoiling and the presence of specific proteins e.g. helix destabilising proteins and single-strand DNA-binding proteins. Certain cellular events, like transcription and DNA replication, may also facilitate the formation or removal of these alternative DNA secondary structures.

1.3.3.5 Inverted repeat sequences and DNA replication

Where the sequence of an origin of replication is known, it is common for palindromic sequences to be found nearby. The presence of palindromes in origin regions is so common in fact, that they have been suggested as a universal feature of the origin locus (Day & Blake, 1982). Palindromic sequences have been found near the origins of replication of *E. coli* K12 (Sugimoto et al., 1979; Meijer et al., 1979); its plasmids (Stalker et al., 1981; Day & Blake, 1982); yeast chromosomal DNA (Tschumper & Carbon, 1982); human mitochondrial DNA (Crewes et al., 1979; Tapper & Clayton, 1981); SV40 virion DNA (Hay & DePamphilis, 1982) and others (Day & Blake, 1982). In some cases the possible alternative secondary structures were proposed as reasons for the presence of the palindromes, but only in single-stranded DNA coliphages like ΦK or G4 has such a suggestion been substantiated. Sims & Benz (1980) showed that the *dnaG* protein (DNA primase) of *E. coli* protected three palindromic regions around the origin of replication from nuclease digestion. Binding of primase to these

regions was specific and appeared to be associated with the adoption of stem-loop structures by the protected sequences.

Another facet of DNA replication in which inverted repeat sequences might play a part is in the replication of the ends of linear DNA molecules by a combination of recombinational and repair events (Cavalier-Smith, 1974). Although such a mechanism has been proposed for replication of the termini of the 61kb linear extrachromosomal rDNA elements of Physarum polycephalum, which are composed of multiple inverted repeats with complex potential secondary structure (Bergold et al., 1983), no direct evidence has yet been found to support this suggestion.

1.3.3.6 Control of gene expression by transcribed inverted repeats

Transcription of inverted repeat sequences produces RNA molecules with obvious potential secondary structure. In this section two mechanisms by which transcription of inverted repeats may control gene expression are described. A feature common to both is the assumption that RNA molecules can and will adopt the secondary structures favoured by their sequence. The examples used are RNA processing by RNAse III and termination of transcription.

RNAse III of E. coli is an enzyme whose major function appears to be one of RNA processing. This enzyme recognises and cleaves RNA molecules at a specific secondary structure consisting of two duplex stems separated by an unpaired loop. RNaseIII is known to be involved in the processing of ribosomal RNA precursors and phage T7 early mRNA (Bram et

al., 1980) and also seems to participate in the control of expression of the int gene of phage λ and the 1.1 and 1.2 genes of phage T7 by selective processing (Gottesman et al., 1982).

Inverted repeat sequences are also found near sites of transcription termination in E. coli (Rosenberg & Court, 1979; Holmes et al., 1983). Typically an RNA molecule ends with a GC-rich inverted repeat sequence followed by a run of several uridylate residues within which termination occurs. Termination appears to be due to the formation of a stem-loop structure by the transcript of an inverted repeat region which causes the RNA polymerase molecule to pause within the adjacent oligo-U run. Enzyme release is initiated by pausing and facilitated by the instability of the dA-rU base pairing. Selective termination within or between genes may be used as a method of control. The presence of multiple inverted repeats within the 5' leader region of certain biosynthetic operons allows selective termination of transcription (attenuation) dependent upon the secondary structures which are allowed to form following transcription of the region (Yanofsky, 1981).

1.3.4 Inverted repeat sequences and prokaryotic differentiation

Although inverted repeat sequences are ubiquitous, their abundance varies from genome to genome. Thus while the DNA of E. coli was found to contain about 0.5% inverted repeat sequences (Kato et al., 1974); three other organisms studied contained much more. In Bacillus subtilis inverted

repeat sequences were found to comprise some 5-10% of the total DNA and have an average size of about 2.5kb (Galloway & Rudner, 1979). Caulobacter crescentus DNA contained 4% inverted repeat ranging from 100-600bp in length (Wood et al., 1976) and Rhodomicrobium vannielii contained approximately 5% inverted repeat of average size 400bp (Potts et al., 1980). As well as possessing large amounts of inverted repeat sequence DNA these three bacteria also exhibit cellular differentiation (as described in section 1.2) and it is possible that their inverted repeat sequences might play a role in this. Alternatively, the abundance of inverted repeat sequences in these organisms might reflect some property of their genomes and have little or no functional importance e.g. G+C content, kinetic complexity, redundancy of coding potential or possession of transposable elements. Another possibility is that these sequences might be an example of "selfish DNA" in prokaryotes having no function beyond their own conservation and propogation. As "selfish DNA" inverted repeat sequences might be more stably conserved than other repeated structures because recombination between the repeats leads merely to inversion of inverted repeats but to deletion of direct repeats.

Table 1.3 shows the G+C content and molecular weights of the genomes of E. coli, B. subtilis, C. crescentus and R. vannielii. From this it appears that the abundance of inverted repeat sequences is unrelated to genome size but may be associated with variations in G+C content away from 50% as observed by Day & Blake (1980). The differences in inverted repeat content found however, seem to be too large to be accounted for by differences in G+C content alone. It

Bacterium	G+C%	Genome Size base pairs	Daltons
E. coli	51	3×10^6	2×10^9
B. subtilis	44	3×10^6	2×10^9
C. crescentus	67	4.5×10^6	3×10^9
R. vannielii	62	3×10^6	2×10^9

Table 1.3 Genome size and G+C content for E. coli, B. subtilis, C. crescentus and R. vannielii. (Adams et al., 1976; Wood et al., 1976; Potts et al., 1980; Henner & Hoch, 1980).

also seems unlikely that these bacteria contain such a large proportion of non-functional "selfish DNA" as this would contradict the bacterial maxim of efficient genetic organisation. Also *E. coli*, which may contain transposons and IS elements, has only 0.5% inverted repeat sequence DNA.

It may then be reasonable to suggest that some of the inverted repeat sequence DNA has some function within these organisms. If so, it is likely that such a function would be common to bacteria with abundant inverted repeats. The most obvious feature which *B. subtilis*, *C. crescentus* and *R. vannielii* share and which is absent in *E. coli* is the ability to initiate and maintain a pathway of cellular differentiation. Such pathways involve temporal and spatial coordination of the expression of many loci with the DNA replication and cell division cycles. The presence of common control elements in association with differentiation-specific genes or operons might provide a relatively simple method for coordinated control of gene expression by one or more of the mechanisms described in section 1.3.3.

The involvement of inverted repeat sequences in the translocation of DNA segments has led to the suggestion that the large scale changes in gene expression observed during bacterial differentiation might be the result of such events. In particular, irreversible changes in gene expression like the maturation of *C. crescentus* or *R. vannielii* swarmer cells; and stable, reversible changes in gene expression like the switch between complex and simplified cell cycles in *R. vannielii* are good candidates for regulation via a recombinational mechanism. Evidence to

support this suggestion has been found for C. crescentus in which a cloned inverted repeat sequence was implicated in a cell cycle associated DNA rearrangement (Nisen et al., 1979). Recent work on B. subtilis and R. vannielli has suggested that in these too, differentiation may be accompanied by rearrangement of specific DNA sequences (Rhaese et al., 1982; Lebens et al., 1982).

DNA rearrangements have also been reported in strains of Streptomyces, another genus of bacteria which exhibits differentiation. Deletion and amplification events have been found in mutants with altered secondary metabolism (Schrempf, 1981; 1982; Fishman & Hershberger, 1983). It is not known, however, whether DNA rearrangements are associated with differentiation.

This project formed an initial investigation of the inverted repeat sequences of R. vannielii. The results presented here report the abundance of these sequences in the R. vannielii genome, their structure, and their distribution in DNA from complex cycle cultures and swarmer cell preparations. The sequence-specific binding of some of these sequences by R. vannielii protein is also described. It is hoped that these results will provide the basis for an investigation of the function of these sequences, particularly during the differentiation of swarmer cells.

CHAPTER 2 MATERIALS and METHODS

The methods described in this chapter are mainly those which were used routinely or preparatively. Those methods used with special reference to certain experiments will be described in the appropriate results section.

2.1 Reagents

All reagents were BDH AnalR grade reagents, except where indicated in the text.

2.2 Bacterial Strains

a. *Rhodomicrobium vannielii* strain 5 (RM5) (Whittenbury & Dow, 1977) was obtained from Dr. C.S. Dow and was used throughout this work.

b. *Escherichia coli* K12 strain HB101 (F^- *pro leu thi lacY* str^R r_K^- m_K^- *recA*; Boyer & Roulland-Doussoix, 1969) was obtained from Dr. N.H.Mann.

c. *Escherichia coli* K12 strain AB259 carrying plasmid pKN80 (Schumann, 1979) was obtained from Dr. W. Schumann.

2.3 Maintenance of bacterial strains

a. *R. vannielii* was grown and maintained in liquid culture in PM medium. In this state cultures retained both

viability and purity for many months. Liquid stock cultures were subcultured every four weeks.

Cells were also stored at -20°C in PM medium plus 50% (v/v) glycerol.

b. E. coli strains were maintained in Nutrient Agar (Oxoid) stab cultures which were renewed quarterly. Cells were also kept in storage at -20°C in LB medium plus 50% (v/v) glycerol.

2.4 Media

a. PM medium (Whittenbury & Dow, 1977) contained:

NH_4Cl	0.50g/l
$MgSO_4 .7H_2O$	0.40g/l
NaCl	0.40g/l
$CaCl_2$	0.05g/l
pyruvic acid sodium salt (Sigma)	1.50g/l
sodium hydrogen malate	1.50g/l

The pH of the medium was adjusted to 6.8 with KOH prior to autoclaving. Before inoculation 50ml/l of sterile 0.1M phosphate buffer pH 6.8 was added aseptically. Solid medium was made by adding Bacto-Agar (Difco) to 1.5% (w/v) before autoclaving.

b. Luria-Bertani (LB) medium contained:

Bacto-Tryptone (Difco)	10g/l
NaCl	10g/l
Yeast Extract (Oxoid)	5g/l
D-glucose	1g/l

Solid medium was made by addition of Bacto-Agar to 1.5% (w/v) before autoclaving.

c. Minimal salts (MS) medium (Hayes & Clowes, 1968) contained:

NH_4Cl	5.0g/l
NH_4NO_3	1.0g/l
Na_2SO_4	2.0g/l
K_2HPO_4	3.0g/l
KH_2PO_4	1.0g/l
$MgSO_4 .7H_2O$	0.1g/l

The contents were dissolved in the order given. A fine white precipitate formed which was removed by filtration before autoclaving. Before inoculation a sterile 50% (w/v) solution of glucose was added to a concentration of 2g/l. Solid medium was made by addition of Bacto-Agar to 1.5% (w/v) before autoclaving.

d. Low-phosphate glucose salts medium (Smith & Midgley, 1973) contained:

NH_4Cl	2.0 g/l
KH_2PO_4	0.002g/l
NaH_2PO_4	0.004g/l
NaCl	3.0 g/l
Na_2SO_4	0.12 g/l
KCl	1.0 g/l
Trizma base (Sigma)	18.17 g/l
$MgCl_2$	0.05 g/l
D-glucose	2.0 g/l

The medium was prepared in two portions; one containing the $MgCl_2$ and D-glucose and the other containing the remaining components. These were autoclaved separately and mixed when cool.

2.5 Sterilisation of media and buffers

In general, all buffers and media were sterilised by autoclaving at 15psi for 15 minutes. Buffers containing labile or volatile components, however, and certain media additives were sterilised by filtration through Millipore GS disposable filter units.

2.6 Spectrophotometry

All spectrophotometric measurements were performed using a PYE Unicam SP1800 recording spectrophotometer.

a. Turbidimetric measurement of culture growth

Measurement of culture absorbance for *R. vannielii* at 540nm ($O.D._{540}$) and for *E. coli* at 600nm ($O.D._{600}$) was used as the main estimate of cell density in liquid cultures.

b. Estimation of nucleic acid concentration

Concentration of DNA in purified samples was measured by absorbance at 260nm ($O.D._{260}$). Contamination by protein and by polysaccharide were estimated by absorbance at 280 and 300nm respectively. Estimation was performed using the relationship that 1 $O.D._{260}$ unit was equivalent to a DNA concentration of 50µg/ml

2.7 Liquid scintillation counting

a. Aqueous samples

^{32}P or ^{3}H radioactivity in aqueous samples of less than 0.1ml was measured directly in 5ml of Beckman 'Cocktail EP' in polypropylene vials in an LKB Mini-Beta liquid scintillation counter with pre-set windows for counting of ^{32}P and ^{3}H isotopes.

b. Samples on filters

Samples were either spotted or filtered onto circular 25mm glass microfibre (Whatman GF/C) or nitrocellulose (Millipore HA) filter discs and allowed to dry for at least 1h at 30°C. Dried filters were placed in polypropylene vials and covered with 5ml of cocktail EP. Radioactivity on

filters was measured three times by liquid scintillation counting, with shaking between counts to take account of errprs caused by filter position.

c. Estimation of radioactivity in agarose gel slices

Slices cut from agarose gels dried onto 3MM filter paper (Whatman), were placed in 3ml glass assay vials and covered with 2.5ml of gel slice scintillant (Benbow et al., 1972).

Gel slice scintillant contains (per litre):

Toluene	858ml
Liquefier	42ml
NCS tissue solubiliser	90ml
H_2O	10ml

Liquefier:

Toluene	42ml
2,5-Diphenyloxazole (PPO)	4g
1,4-Di-2(5-Phenoxazolyl) Benzene (POPOP)	50mg

Capped vials were incubated at 37°C for 48h, care being taken to ensure that vial caps were not allowed to come off. Vials were cooled to room temperature before scintillation counting as described above.

2.8 Growth and differentiation of R. vannielii

a. Culture of R. vannielii

R. vannielii was grown in liquid batch cultures of up to 20l in PM medium as described by Whittenbury & Dow

(1977). Culture vessels were capped with rubber serum caps (Suba-seals); flushed with oxygen-free nitrogen (BOC) and inoculated with 1/100th of a volume of a liquid stock culture. Incubation was at 30°C with lighting from tungsten filament bulbs of approximately 2000lux and with constant stirring.

b. Selection of homogeneous swarmer cell populations

A complex cycle culture in late logarithmic growth ($O.D._{540}$ 0.8-1.0) was incubated overnight in the dark at 30°C to maintain swarmer cell numbers. Swarmer cells were selected by passage through a glass wool column into a darkened receiving vessel which had been flushed with nitrogen. On completion of the selection process the receiving vessel was made air-tight and incubated in the dark for 1h before initiation of differentiation. Such synchronous swarmer cell populations may be held in the dark for up to 16h without loss of viability (Dow et al., 1983).

c. Differentiation of synchronous swarmer cell populations

Differentiation of swarmer cells was initiated by re-illumination of homogeneous swarmer cell populations under standard growth conditions. Swarmer cell differentiation was followed microscopically and by changes in cell volume. Cell volume distribution profiles were generated by a ZB1 Coulter Counter and a C1000 Channelyser (Coulter Electronics Ltd., Harpenden, Herts.) linked to a Commodore PET 8032 microcomputer or BBC model B microcomputer. By this method differentiation was found to continue synchronously for at least 6h.

2.9 Preparation of R. vannielli DNA

a. Cell Lysis

R. vannielli is relatively resistant to lysis by conventional lysozyme/EDTA treatment, except at cellular growth points (Dow et al., 1983). Therefore use of such a method for isolation of chromosomal DNA from R. vannielii would extract DNA only from that portion of the cell population possessing growth points. To ensure that uniform lysis throughout the cell population was obtained, the following method was developed.

Cells, either from heterogeneous cultures or from differentiating swarmer cell preparations, were harvested by centrifugation at 23,000 xg for 20 minutes. Cell pellets were washed once in TES buffer (0.05M Tris/HCl pH 8.0, 0.005M Na_2EDTA, 0.05M NaCl) and resuspended in TES buffer containing 25% (w/v) sucrose. Washed cells were passed through a french pressure cell at 1000psi. This step caused minimal lysis (as assessed by microscopy) but rendered the cells uniformly sensitive to lysis by lysozyme/EDTA treatment. Lysozyme (Sigma) was added to a final concentration of 1mg/ml and the cell suspension incubated at 37°C for 10 minutes. This was followed by addition of Na_2EDTA to 0.05M, transfer to ice and addition of sodium N-lauroylsarcosinate (Koch-Light Laboratories) to 3.5% (w/v). The lysate was then made 1M with respect to sodium perchlorate and incubated for 10 minutes at 65°C.

b. Purification of DNA

The cell lysate, obtained as described above, was

deproteinised twice by extraction with phenol/ chloroform/amyl alchohol (25:24:1, w/w) saturated with TE buffer (0.01M Tris/HCl pH 7.5, 0.001M Na_2EDTA) and the aqueous phase was retained. To this two volumes of absolute ethanol were added and nucleic acids allowed to precipitate at -20°C for 16h. The nucleic acid pellet, obtained by centrifugation, was dissolved in 5ml of TE buffer and treated with 20µg/ml of Ribonuclease (RNAse) A (Sigma) and 100units/ml of RNAse T1 (Sigma) for 30 minutes at 37°C. After RNAse treatment the volume of the DNA solution was made up to 30ml with TE buffer and 32g of CsCl added. The solution was then divided between two 25ml screw cap polycarbonate ultracentrifuge tubes (MSE) and centrifuged at 100,000 xg for 48h at 20°C.

The CsCl density gradients formed were fractionated from below and DNA-containing fractions detected by spotting 5µl aliquots of each fraction onto a plate of 1.0% (w/v) agarose (Type II, Sigma) containing 1µg/ml ethidium bromide (Sigma). Fractions which showed fluorescence in ultraviolet light were pooled and dialysed against TE buffer for 24h with 3 changes of buffer. Dialysed DNA was decanted into sterile bijou bottles and stored at 4°C over chloroform.

2.10 Preparation of E. coli DNA

Lysis of *E. coli* cells was obtained by the method described in section 2.9a for *R. vannielii* except that the french press and 65°C incubation steps were omitted.

CsCl gradient purification of *E. coli* DNA and its

subsequent storage were performed exactly according to the procedure described in section 2.9b.

2.11 Preparation of plasmid DNA from E. coli

a. Growth of cells

One litre cultures of E. coli strains carrying plasmids were grown in LB containing the appropriate antibiotic(s) for the plasmid carried. Cultures were incubated at 37°C and were allowed to reach stationary phase before plasmid isolation.

b. Preparation of cleared lysates

Cleared lysates of E. coli cultures were prepared by the method of Katz et al. (1973) as follows:

1. Cells were pelleted by centrifugation at 23,000 xg for 10 minutes.
2. Cell pellets were resuspended in a final volume of 12.5ml of 0.05M Tris/HCl pH 8.0 containing 25% (w/v) sucrose in a 30ml Oak Ridge centrifuge bottle (MSE).
3. 2.5ml of fresh 10mg/ml lysozyme in 0.01M Tris/HCl pH 8.0 was added and the suspension was held on ice for 5 minutes.
4. 2.5ml of 0.5M Na_2EDTA pH 8.0 was then added and the mixture was swirled gently on ice for 2-3 minutes.
5. To this 20ml of a solution containing 0.05M Tris/HCl pH 8.0, 0.062M Na_2EDTA pH 8.0, 0.1% (v/v) Triton X-100 (Koch-Light Laboratories) was added and mixed gently by inversion.
6. The lysis mixture was then centrifuged for 45 minutes at

40,000 xg and the resulting supernatant retained.

c. Purification of plasmid DNA

Nucleic acids were precipitated from the cleared lysate by addition of 1/2 volume of 30% (w/v) polyethylene glycol (average Mr. 6000) in 1.5M NaCl, and incubation on ice for 30 minutes.

Pellets obtained by centrifugation at 15,000 xg for 15 minutes were drained thoroughly and redissolved in 5ml of TE buffer. RNAse A was then added to 20µg/ml and the solution incubated at 37°C for 30 minutes. The volume of the solution was made up to 29ml with TE buffer, and 1ml of 5mg/ml ethidium bromide and 30g of CsCl were added. When dissolved the CsCl/DNA/TE solution was split between two 25ml screw cap polycarbonate tubes and centrifuged at 100,000 xg for 48h at 20°C.

After centrifugation DNA bands were visualised by ultraviolet fluorescence of DNA-bound ethidium bromide. The lower, plasmid band was collected and the bound ethidium bromide was removed by repeated extraction with CsCl/TE saturated isopropanol. The plasmid DNA was then dialysed against TE buffer and stored at 4°C as above (section 2.9b).

d. Rapid, small-scale plasmid preparation

Plasmid preparations from between 1ml and 5ml of culture were performed by the method described by Ish-Horowicz and Burke (1981) as follows:
1ml of an overnight culture was harvested by centrifugation in a 1.5ml microcentrifuge tube. The cells were resuspended in 100µl of 0.05M glucose, 0.025M Tris/HCl pH 8.0, 0.01M

Na_2EDTA and were incubated for 5 minutes at room temperature before adding 200µl of 0.2N NaOH, 1% (w/v) sodium dodecyl sulphate (SDS) and mixing gently by inversion. The resulting lysate was held on ice for 5 minutes and then 150µl of cold 5M potassium acetate pH 4.8 was added and mixed gently. Cell debris and precipitated chromosomal DNA were pelleted by centrifugation for 1 minute in a microcentrifuge and the supernatant transferred to a clean tube. Two volumes of absolute ethanol were added and plasmid DNA was allowed to precipitate for two minutes at room temperature. The precipitate was pelleted by centrifugation as before; the pellet washed with 70% (v/v) ethanol, dried under vacuum and redissolved in a small volume of TE buffer.

2.12 In vivo incorporation of ^{32}P orthophosphate into R. vannielii DNA

It was found that R. vannielii was able to grow in PM medium without added phosphate buffer with a 1% (v/v) inoculum from a culture grown under normal conditions as its sole source of phosphate, when buffered by 0.005M Tris/HCl pH 6.8. Cell morphology appeared unchanged under these growth conditions, but the final cell density attained by such cultures was approximately half that of cultures grown in normal PM medium.

Addition of carrier-free ^{32}P-orthophosphate (PBS11, Amersham; 10mCi/ml) to low phosphate cultures to a final concentration of 10µCi/ml produced significant incorporation

of ^{32}P into chromosomal DNA. Labelled DNA was extracted and purified as described in section 2.9.

2.13 In vivo incorporation of ^{3}H-adenosine into R. vannielli DNA

R. vannielii DNA was labelled with ^{3}H-adenosine as described by Potts (1980).

^{3}H-adenosine (Amersham; specific activity 20-25 Ci/mmol) was added to PM medium to a concentration of 5μCi/ml. The final concentration of adenosine in the culture was made 0.005M by addition of unlabelled adenosine (Sigma). The culture was inoculated and grown as described (section 2.8) until late logarithmic phase of growth was reached. The cells were then harvested and DNA extracted as described above (section 2.9).

2.14 In vivo incorporation of ^{32}P-orthophosphate into E. coli DNA

E. coli K12 strain HB101 was grown in low-phosphate glucose salts medium with added L-proline and L-leucine present at 100μg/ml and thiamine at 20μg/ml. ^{32}P-orthophosphate was added to a final concentration of 1μCi/ml. The culture was allowed to reach early stationary phase before extraction and purification of DNA as described above (section 2.10).

2.15 Restriction Endonuclease digestion of DNA

All restriction endonucleases were used according to the suppliers instructions, reactions normally being done with twice the required amount of enzyme to ensure complete digestion.

The DNA to be digested was first precipitated with ethanol in the presence of 100µg glycogen as carrier (DNA-, RNA- and nuclease-free, purified as described in section 2.23). The precipitate was collected by centrifugation, washed once with 100µl of 70% (v/v) ethanol, and dried under vacuum. The DNA pellet was then redissolved in 50µl of the appropriate buffer and the restriction enzyme added. After digestion at 37°C the extent of digestion was checked by electrophoresis of a portion of the digestion on a minigel apparatus.

2.16 Gel electrophoresis of DNA fragments

a. Agarose gels

Agarose gels of up to 3% (w/v) were used for fractionation of DNA fragments. Agarose (type II, Sigma) was dissolved in running buffer to the required concentration by autoclaving and gels were poured after cooling to 50°C. Gels were allowed to set for at least 1h before use.

For horizontal gels Tris-acetate (TEA) buffer was used (Helling et al., 1974). Stock buffer was prepared as a x10 concentrate.

Stock TEA contained:

Tris	19.38g/l
Na acetate .$3H_2O$	10.89g/l
Na_2EDTA	2.98g/l
NaCl	4.68g/l

pH was adjusted to 8.0 with glacial acetic acid.

For vertical gels Tris-borate (TBE) buffer was used (Peacock & Dingman, 1968). Stock buffer was prepared as a x10 concentrate. Stock TBE contained:

Tris	108g/l
boric acid	55g/l
Na_2EDTA	9.3g/l

pH was 8.3 without adjustment.

b. Polyacrylamide gels

Vertical polyacrylamide gels of 5-8% (w/v) were made by dilution of a stock solution of 30% (w/v) acrylamide monomer plus 1% (w/v) N,N'-methylenebisacrylamide (Eastman Kodak) into running buffer (TBE). The solution was degassed under vacuum and immediately prior to casting the gel 100µl of 10% (w/v) ammonium persulphate and 30µl of N,N,N',N'-tetramethylethylenediamine (TEMED) were added. Gels were cast between 20x25cm glass plates separated by 1mm thick plastic spacers and sealed with silicone rubber tubing. When set, the gel was overlaid with a 4% stacking gel made in the same way and a sample well comb was inserted. Gels were left to mature overnight before use.

c. Electrophoresis

Horizontal agarose gels were run at 12mA constant current for up to 16h.

Vertical agarose or polyacrylamide gels were run at 50mA constant current for 2-4h. Care was taken to ensure that the gels did not overheat during electrophoresis.

Gels were stained by soaking in a 1µg/ml solution of ethidium bromide (EtBr) for up to 1h. Destaining was done in running buffer or water for a further hour. Stained DNA bands were visualised by fluorescence of bound EtBr when illuminated on a long-wave ultraviolet transilluminator (Shandon Southern). Gels were photographed using a Polaroid Land Camera XL MP3 with ultraviolet filters (Ilford Nos. 110 & 805) and type 665 positive/negative Polaroid Land Pack film.

Alternatively, gels containing radioactive DNA fragments were dried onto Whatman 3MM filter paper and autoradiographed in an X-ray cassette (Harmer) with intensifying screens (DuPont Cronex lightning plus) and X-ray film (Fuji RX) at -70°C. Autoradiographs were developed with Kodak DX-80 developer and fixed using Kodak FX-40 fixer.

2.17 Transfer of DNA fragments from agarose gels to nitrocellulose filters

Blot tranfer of DNA fragments from gels to membrane filters was performed according to the method of Southern (1975).

After photographing the gel as described in section 2.16c DNA fragments were denatured *in situ* by soaking in a solution of 1.5M NaCl, 0.5M NaOH for 30 minutes followed by neutralisation in 3M NaCl, 0.5M Tris/HCl pH 7.0 for 30 minutes. The gel was then trimmed to 12x15cm and laid on a layer of 3MM filter paper which had been soaked in 20xSSC (1xSSC is 0.15M NaCl, 0.015M Na_3Citrate pH 7.0) and both ends of which were folded down into a reservoir of 20xSSC. The gel was surrounded by spacers of the same height as the gel, leaving a 5mm gap between gel and spacers on all sides. A 14x17cm nitrocellulose membrane filter (Schleicher & Schull BA85) was wetted with 2xSSC and carefully placed on the gel so as to exclude air bubbles and to rest on the spacers around the gel. The filter was covered with two 14x17cm sheets of 3MM paper soaked in 2xSSC and on these was placed a stack of absorbant paper of the same size. A weight was placed on top of the apparatus to assist transfer which was allowed to continue for 16h. The assembled transfer apparatus is shown schematically in figure 2.1.

After transfer the apparatus was disassembled and the nitrocellulose filter recovered. The filter was rinsed in 2xSSC, blotted dry, and baked for 2h at 80°C in a vacuum oven. Baked filters were stored dry in foil or plastic envelopes.

2.18 Hybridization of radioactive DNA to DNA immobilised on nitrocellulose filters

Hybridization was carried out by a modification of the method of Denhardt (1966). Filters carrying bound DNA were

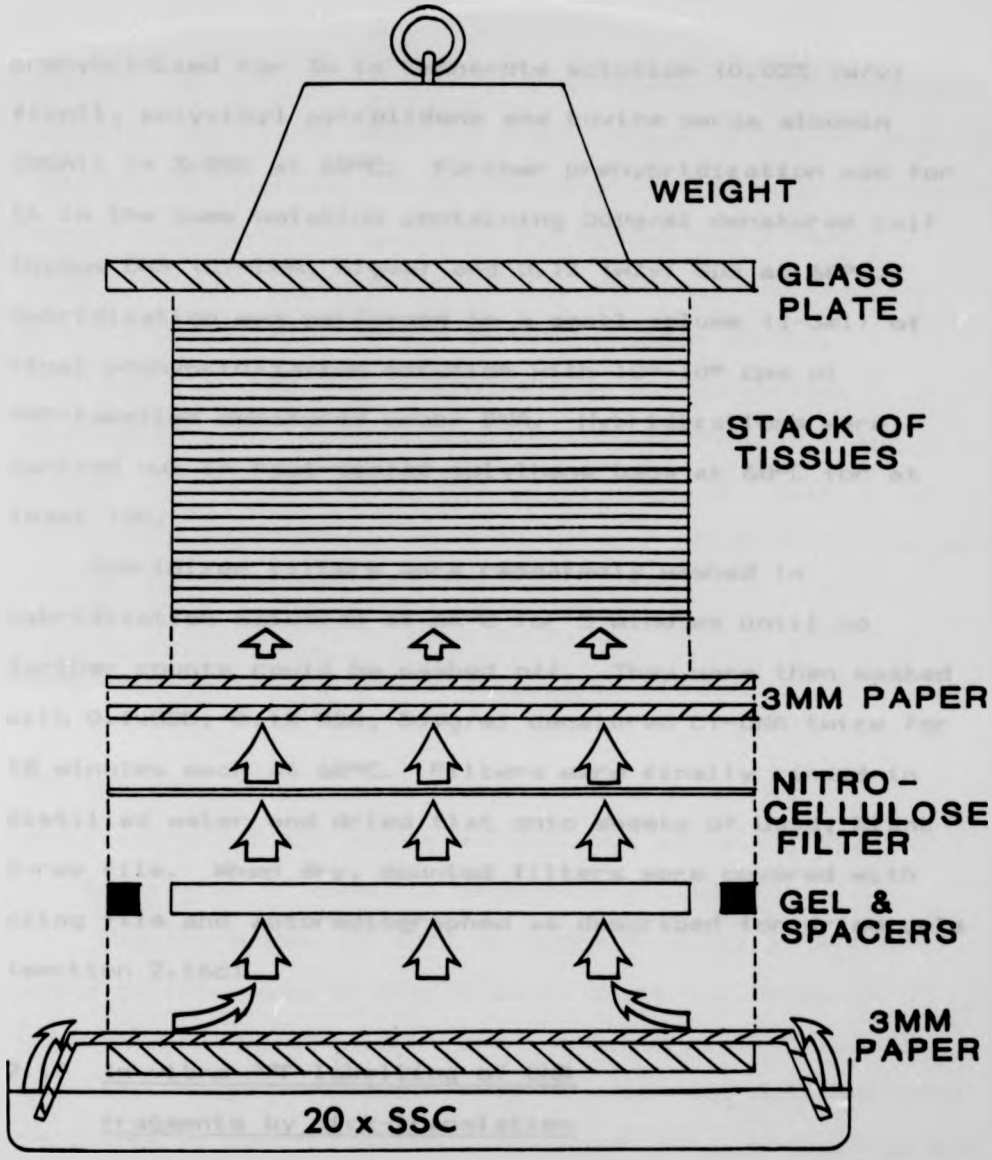


Figure 2.1 Apparatus used for "Southern" transfer of DNA fragments from agarose gels to nitrocellulose filters. Open arrows indicate the direction of buffer flow.

prehybridized for 3h in Denhardts solution (0.02% (w/v) ficoll, polyvinyl pyrrolidone and bovine serum albumin (BSA)) in 3xSSC at 68°C. Further prehybridization was for 1h in the same solution containing 50µg/ml denatured calf thymus DNA (CT-DNA, Sigma) and 0.1% (w/v) SDS at 68°C. Hybridization was performed in a small volume (1-3ml) of final prehybridization solution with 10^4–10^6 cpm of ^{32}P-labelled denatured probe DNA. Hybridizations were carried out in heat-sealed polythene bags at 68°C for at least 16h.

Hybridized filters were repeatedly washed in hybridization solution at 68°C for 5 minutes until no further counts could be washed off. They were then washed with 0.1xSSC, 0.1% SDS, 50µg/ml denatured CT-DNA twice for 15 minutes each at 68°C. Filters were finally rinsed in distilled water and dried flat onto sheets of used, blank X-ray film. When dry, mounted filters were covered with cling film and autoradiographed as described for dried gels (section 2.16c).

2.19 In vitro ^{32}P-labelling of DNA fragments by nick-translation

Nick-translation (Rigby et al., 1977) was performed using α^{32}P-dGTP or α^{32}P-dCTP (Amersham; specific activity 3000 Ci/mmol at a concentration of 10µCi/µl) as follows:

An equal volume of the labelled deoxynucleoside triphosphate and 10µM stock solutions of the remaining three unlabelled triphosphates (Sigma) were added to the substrate DNA (up to 1µg) and 4µl of 5xNT buffer (5xNT buffer is 0.25M

Tris/HCl pH 7.5, 0.05M $MgCl_2$, 0.05M Dithiothreitol (DTT, Sigma)). Water was added to give a final volume of 19µl and the reaction mixture was preincubated at 14°C for 15 minutes before addition of 5 units of DNA polymerase 1 (BRL). Incubation was continued for 3h at 14°C and the reaction stopped by addition of 1µl of 0.5M Na_2EDTA pH 8.0.

Labelled DNA was separated from unincorporated deoxynucleoside triphosphates by passage through a 2ml column of Sephadex G-50 (Pharmacia) which had been previously equilibrated with TE buffer. Peak fractions were pooled and stored at 4°C.

2.20 End-labelling of DNA fragments

DNA fragments were end-labelled with γ^{32}P-ATP by a modification of the method of Maniatis et al. (1982).

a. Removal of 5' terminal phosphate groups

Substrate DNA, in 80µl of 0.05M Tris/HCl pH 8.0 was added to 100 units of Matrix-bound Bacterial Alkaline Phosphatase (BRL MATE-BAP) and incubated at 37°C for 30 minutes. After incubation substrate DNA and MATE-BAP were separated by centrifugation. MATE-BAP was washed twice and stored at 4°C in 0.01M Tris/HCl pH 8.0, 0.1M NaCl, 0.02% (w/v) sodium azide. Substrate DNA was ethanol precipated with 100µg glycogen as carrier.

b. Polynucleotide kinase reaction

Phosphatase-treated DNA in 30µl of TE buffer was added to 5-10µCi of γ^{32}P-dATP, 3.5µl of 10xPNK buffer (10xPNK buffer was 0.5M Tris/HCl pH 7.6, 0.1M $MgCl_2$, 1.25M NaCl, 0.05M DTT, 0.01M spermidine hydrochloride (Sigma)) and 1µl

of T4 polynucleotide kinase (BRL). The reaction mixture was incubated for 30 minutes at 37°C and the reaction stopped by incúbation at 65°C for 5 minutes followed by chilling on ice. Unincorporated nucleotides were separated from labelled DNA as described in section 2.19.

2.21 Preparation of DNA-free, cell-free extracts from R. vannielii

R. vannielii cells from complex cycle cultures or differentiating swarmer cell preparations were harvested by centrifugation and treated as follows:

Cell pellets were resuspended in a buffer containing 0.02M Tris/HCl pH 7.5, 0.01M $MgCl_2$, 0.002M $CaCl_2$, 0.001M Na_2EDTA, 0.001M 2-mercaptoethanol (Sigma) and were broken at 4°C by repeated passage through a French pressure cell at 20,000 psi until over 95% breakage was obtained as determined microscopically. The resulting cell-free extract was treated with DNAse 1 (Sigma) at 10µg/ml for 1h at room temperature before removal of the particulate fraction by centrifugation at 75,000 xg for 1h. The supernatant was retained and dialysed against 0.02M Tris/HCl pH 7.5, 0.05M NaCl, 0.001M Na_2EDTA, 0.001M 2-mercaptoethanol at 4°C for 24h with 3 changes of buffer. Extracts were decanted and made 10% (w/w) in glycerol before drop-freezing in liquid nitrogen and storing at -70°C. Protein content in the extracts was determined using the Bio-Rad standard protein assay (Bio-Rad Laboratories) with lysozyme as a standard.

2.22 Transformation of E. coli with plasmid DNA

E. coli cells were made competent and transformed with plasmid DNA by the following method (Holland, 1983).

An overnight culture of the recipient *E. coli* strain was diluted 1/50 into prewarmed LB medium and incubated with fast shaking at 37°C until the culture $O.D._{600}$ reached 0.5-0.6. The cells were then harvested by centrifugation, resuspended in 1/2 volume of cold, sterile 0.1M $MgCl_2$, spun down again, resuspended in 1/2 volume of cold, sterile 0.1M $CaCl_2$, spun down a third time and finally resuspended in 1/20 volume of cold, sterile 0.1M $CaCl_2$ after which they were held on ice for 1h. The plasmid DNA was then added in 100µl of 0.1M $CaCl_2$ to 200µl of competent cells. The transformation mixture was left on ice for 1h, heat-shocked at 42°C for 2 minutes, left on ice for a further 30 minutes and then incubated at 37°C in LB medium. After 90 minutes incubation without selection the transformed cells were used to inoculate solid and liquid selective media.

2.23 Purification of glycogen for use as co-precipitant

Oyster glycogen (Sigma) was made DNA-, RNA-, and protein-free by the following method (M.R. Lebens; personal communication).

Glycogen (5g) was dissolved in 50ml of DNAse buffer (0.1M Tris/HCl pH 7.5, 0.05M NaCl, 0.002M $MgCl_2$) and DNAse 1 was added to a final concentration of 10µg/ml. After 1h at

room temperature RNAse A was added to 50µg/ml and incubated at 37°C for 30 minutes. The solution was then treated overnight with 5µg/ml of proteinase K (Sigma). The nucleic acid-free glycogen was made 0.3M in NaOH and incubated for 2h at 65°C, neutralised with 0.3M HCl, deproteinised with an equal volume of phenol/chloroform/amyl alchohol (25/24/1, w/w) and the aqueous phase extracted with chloroform before precipitation with 3 volumes of ethanol at -20°C overnight. The precipitate was collected by centrifugation and dried under a stream of nitrogen. Purified glycogen was stored at -20°C as solid or as a 10mg/ml solution in water.

2.24 Gel electrophoresis of proteins

Proteins were electrophoresed in 5-20% polyacrylamide gradient gels using the discontinuous buffer system of Laemlii (1970). Gradient gels were formed by continuous mixing of 5% and 20% (w/v) acrylamide stocks (containing 0.1% (w/v) N,N'-methylene bisacrylamide) in 125mM Tris/HCl pH 8.0, 0.1% (w/v) SDS, 0.1% (w/v) linear polyacrylamide in a two-chambered gradient former. Gels were 20 x 25cm in size and 1mm thick. A stacking gel of 4% (w/v) acrylamide in 125mM Tris/HCl pH 6.8, 0.1% SDS was cast on top of the main gel and sample wells were formed in this. Protein samples of about 100µg were prepared for electrophoresis by incubating at 100°C in 125mM Tris/HCl pH 7.5, 10% (w/v) sucrose, 4% (w/v) SDS and 10mM 2-mercaptoethanol for two minutes. Protein size standards (Pharmacia) were similarly treated. After loading, gels were electrophoresed for up to

24h at 15mA. Gels were fixed by the method of Steck et al. (1980) and were stained with silver nitrate by the method of Wray et al. (1981) using the modifications described by Eschenbruch & Burk (1982) as follows. After electrophoresis, gels were fixed by soaking for 1h in 500ml of formaldehyde/ethanol/water (1/2/4, v/v). They were then prepared for silver staining by soaking in 50% (v/v) SLR (standard laboratory reagent grade) methanol overnight followed by three alternate water and 50% methanol 30 minute washes. The silver stain was prepared by dropwise addition of 4ml of 20% (w/v) silver nitrate to a mixture of 21ml 0.36% (w/v) NaOH and 1.4ml 14.8M NH_4OH. When all of the silver nitrate solution had been added the volume of the staining solution was made up to a total of 100ml with water. Gels were soaked in the silver stain solution for 30 minutes with gentle shaking. The stain was then removed and the gel washed 3 times for 5 minutes each in distilled water. The developing solution was made by addition of 0.25ml of 38% formaldehyde solution to 2.5ml of 1% (w/v) citric acid. This was then made up to 500ml with water and was used immediately. Silver stained bands were found to develop during the 10-30 minute period after addition of developer. When the gels were satisfactorily stained they were washed in water and stored in 45% methanol/10% (v/v) acetic acid. Gels were photographed by transmitted light on Kodak Panatomic-X film using a Pentax K-1000 camera.

Chapter 3 Results and Conclusions

3.1 Introduction

In this chapter the experiments used to investigate the abundance, structure and function of the inverted repeat sequences of R. vannielii will be described. For each set of experiments the methods used, results obtained and conclusions drawn will be presented. Also described are two experiments aimed at determining if any DNA rearrangements occur associated with swarmer cell differentiation and an initial investigation of the serine proteases in differentiating swarmer cells.

3.2 R. vannielii DNA: isolation and restriction analysis

3.2.1 DNA Extraction

The observation that R. vannielii cells are considerably more sensitive at their growth points to lysis by either physical or enzymatic means (Dow et al., 1983) suggested that in order to efficiently obtain DNA from all cell types in a heterogenous culture an appropriate method of DNA extraction was required. This method should be capable of extracting DNA from all of the cell types in a complex culture with high yield. The protocol described in section 2.9 is the best such method presently available. It is based on conventional lysozyme/EDTA lysis methods (e.g. Smith, 1967) with a number of additional steps to ensure maximal and uniform cell lysis. By this method yields of 1mg DNA per litre of culture were obtained routinely.

3.2.2 Restriction enzyme analysis of total DNA

R. vannielii DNA obtained from complex cycle cultures was digested with the restriction endonucleases *Eco*RI, *Hind*III, *Bam*HI and *Pst*I and the digested DNA analysed by electrophoresis in 0.7% (w/v) agarose horizontal slab gels as described in section 2.16. The characteristic digestion patterns produced are shown in the appropriate lanes of figures 3.1 and 3.2. Densitometer analysis of *Hind*III digested DNA (not shown) resolved over 85 distinct bands.

DNA from inhibited (0h) and differentiated (6h) swarmer cells was also analysed using the same four enzymes. In each case the swarmer cell DNAs exhibited no discernable differences from the complex cycle DNA (figures 3.1 & 3.2)

The gross methylation of *R. vannielii* DNA was examined using the restriction enzyme isoschizimers *Hpa*II and *Msp*I. *Hpa*II cleaves at the sequence 5'-CCGG-3' only if the second cytosine residue is unmethylated, while *Msp*I cuts at the same sequence whether or not it is methylated. Little difference can be observed between the digestion patterns produced by *Hpa*II and *Msp*I (not shown), suggesting that there is no large-scale methylation of the sequence CCGG at any stage in the *R. vannielii* cell cycle but this does not rule out methylation of specific sites within the genome.

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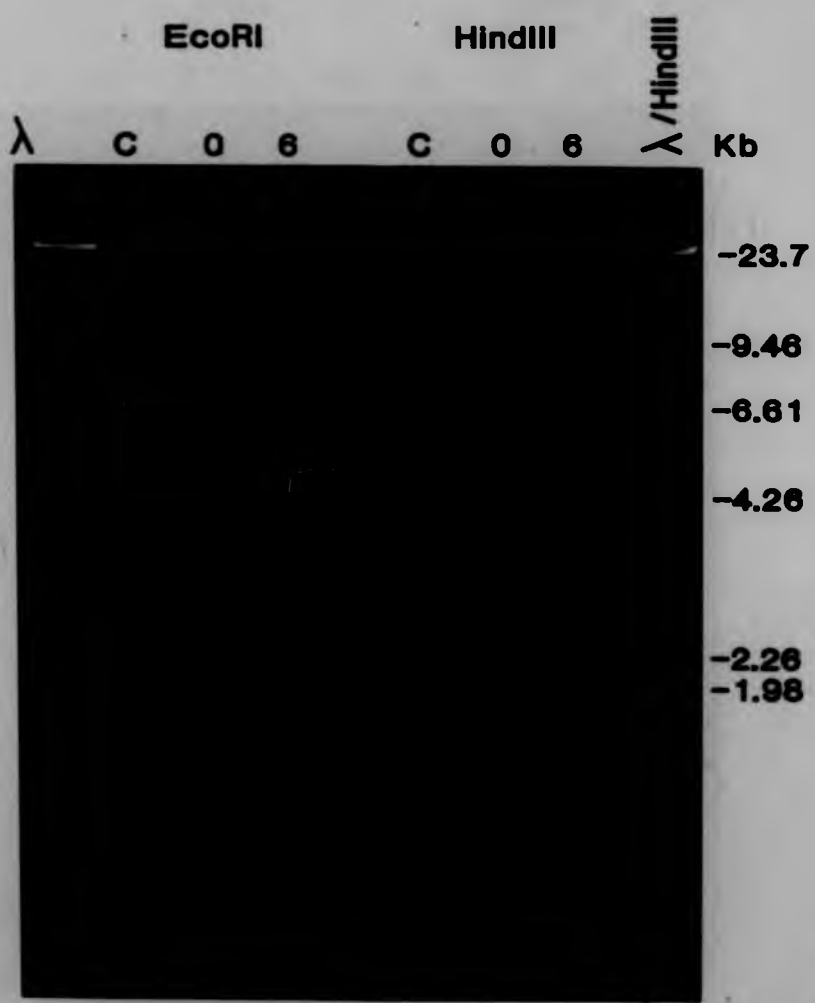
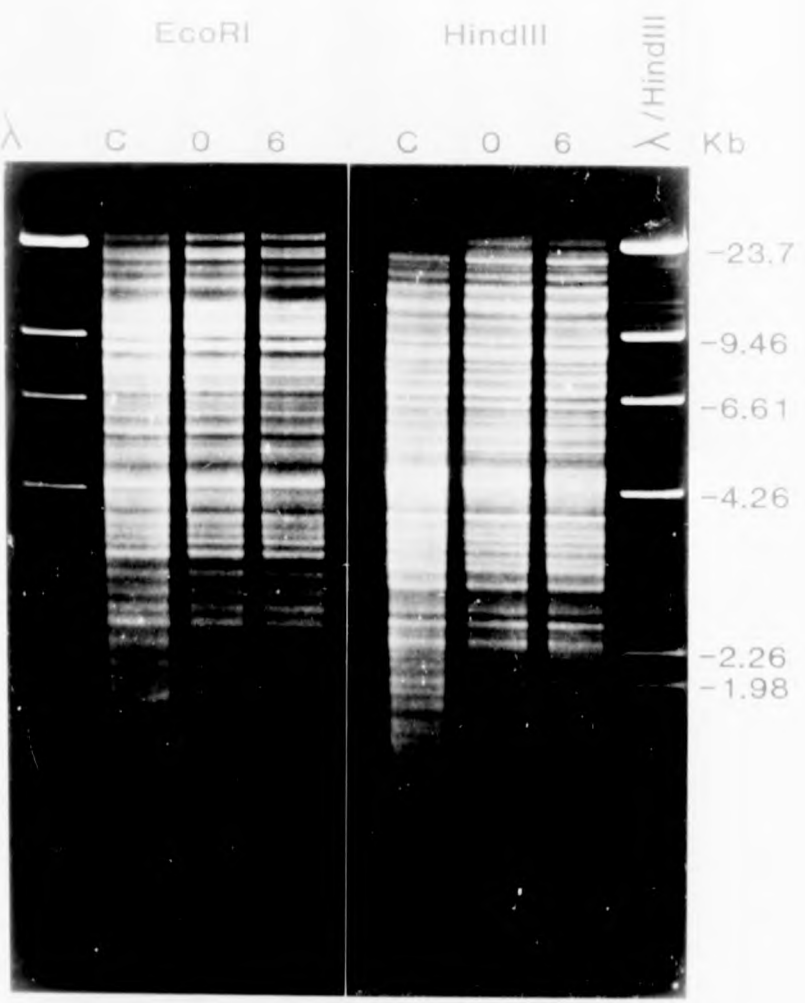
Figure 3.1 Ethidium Bromide (EtBr) stained 0.7% agarose gel of EcoRi and HindIII restriction enzyme digestions of R. vannielii chromsomal DNA (5µg each) from complex cycle (C), 0h swarmer (0) and 6h swarmer (6) cells. Size standards are bacteriophage λ DNA (1µg) digested with HindIII, fragment sizes as indicated.
Note that the topmost bands in each lane may be of unequal intensity due to variations in the fragment sizes of the chromosomal DNA preparations used.

Figure 3.1 Ethidium Bromide (EtBr) stained 0.7% agarose gel of EcoR1 and HindIII restriction enzyme digestions of R. vannielii chromsomal DNA (5µg each) from complex cycle (C), 0h swarmer (0) and 6h swarmer (6) cells. Size standards are bacteriophage λ DNA (1µg) digested with HindIII, fragment sizes as indicated.
Note that the topmost bands in each lane may be of unequal intensity due to variations in the fragment sizes of the chromosomal DNA preparations used.


EcoRI
HindIII
λ/HindIII
λ C 0 6 C 0 6 λ
Kb
-23.7
-9.46
-6.61
-4.26
-2.26
-1.98


EcoRI
HindIII
λ/HindIII
λ C 0 6 C 0 6 λ Kb
23.7
9.46
6.61
4.26
2.26
1.98

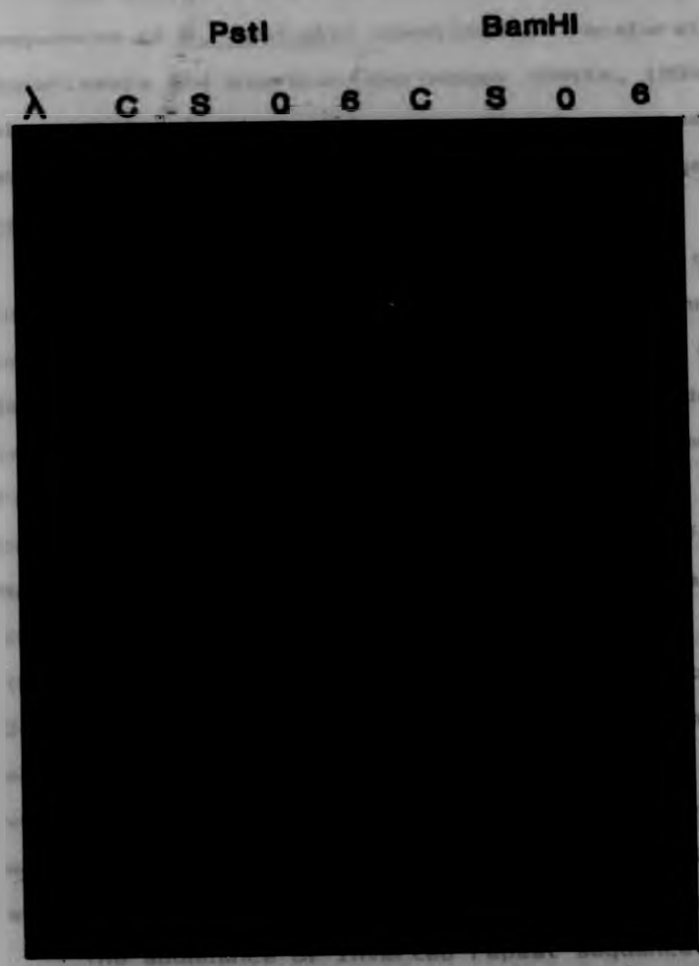
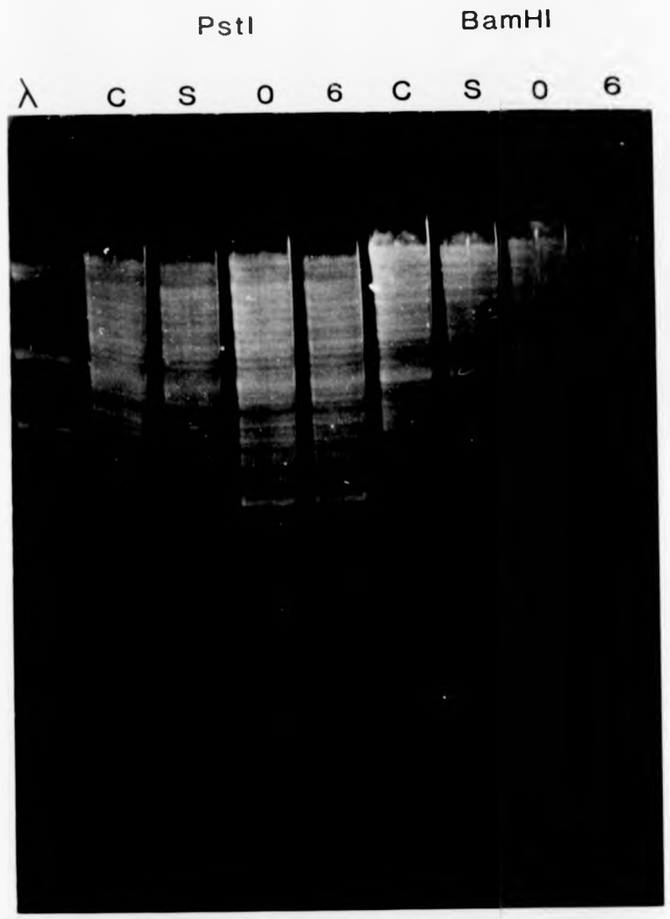


Figure 3.2 EtBr stained 0.7% agarose gel of PstI and BamHI restriction enzyme digestions of R. vannielii chromsomal DNA from complex cycle (C), simplified cycle (S), 0h swarmer (0) and 6h swarmer (6) cells. Size standards are as figure 3.1.


PstI
BamHI
λ C S 0 6 C S 0 6

3.3 Abundance of inverted repeat sequence DNA in R. vannielii and E. coli

The initial characterisation of the inverted repeat sequences of R. vannielii consisted of renaturation experiments and electron microscopy (Potts, 1980; Potts et al., 1980). In this work a more direct measurement of the abundance of inverted repeat sequences in the genome of R. vannielii was attempted.

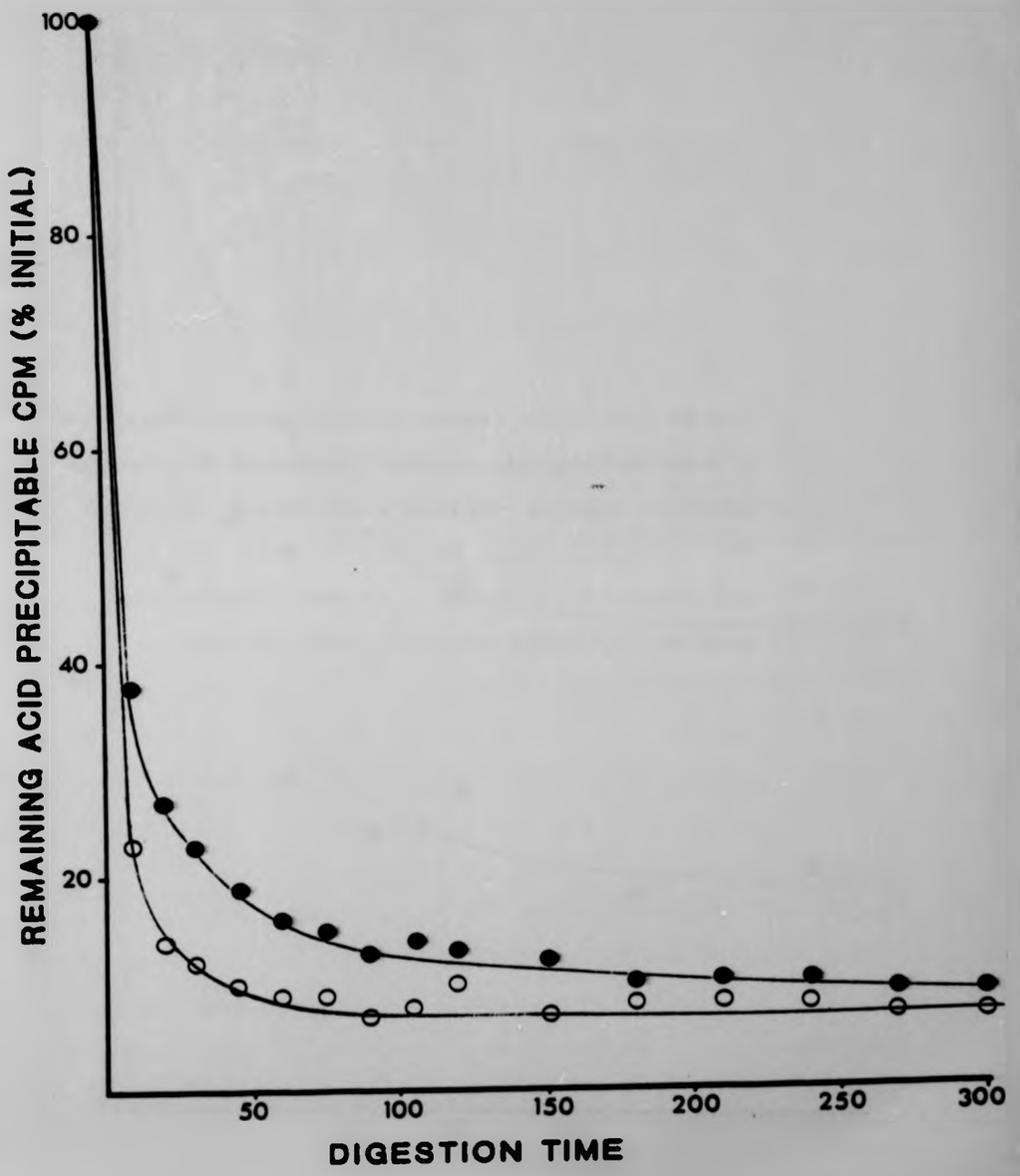
Inverted repeat sequence DNA (IR DNA) was obtained by digestion of denatured and rapidly renatured chromosomal DNA with the single-strand specific nuclease S1 as follows: DNA at a concentration of 1mg/ml in water was denatured by incubating at 100°C for 10 minutes followed immediately by rapid chilling on ice, yielding 0Cot renatured DNA. When cool an equal volume of x2 concentrated S1 buffer (0.6M NaCl, 0.06M Na acetate, 0.009M $ZnCl_2$ pH 4.6) was added to the 0Cot renatured DNA and 1 unit/µg DNA of S1 nuclease (BRL) was then added. Digestion was allowed to continue for 2-3h at 37°C, after which the reaction was stopped by additon of Na_2EDTA to 30mM and extraction with an equal volume of phenol/chloroform/amyl alcohol. S1-resistant DNA was then precipitated at -20°C by addition of 2 volumes of ethanol in the presence of 100µg glycogen as carrier.

The abundance of inverted repeat sequences was determined by assaying trichloroacetic acid (TCA) precipitable DNA throughout S1 digestion of denatured and 0Cot renatured ^{32}P-labelled chromosomal DNA. Nuclease S1 digestion of ^{32}P-labelled DNA (10^4—10^7 cpm/assay) was carried out as described above. Duplicate 5µl samples were taken at various times during digestion and assayed as follows:

Samples were added to 500µl of Calf Thymus DNA (200µg/ml) in TE buffer on ice. They were then made 10% (w/v) in TCA and were left on ice for 30 minutes before collecting precipitable material by filtration on 25mm glass microfibre filter discs (Whatman GF/C). The filters were washed with 3x1ml of 5% (w/v) TCA and 1ml of ethanol, air-dried at 30°C and assayed for acid-precipitable counts by scintillation counting as described in section 2.7b.

In parallel experiments this method was used to determine the abundance of S1 resistant OCot DNA in the genomes of E. coli K12 and R. vannielii. The time courses of nuclease S1 digestion are shown in figure 3.3. These reveal that 9% of R. vannielii DNA and 7% of E. coli DNA is resistant to S1 digestion after denaturation and rapid renaturation. In each case the S1 resistant fraction was isolated and subjected to a further round of denaturation, renaturation and S1 digestion. This should remove all of the inverted repeat DNA as S1 nuclease can cleave even at the smallest hairpin loops (Lilley & Markham, 1983) leaving duplex DNA molecules which should not renature spontaneously upon denaturation and cooling (figure 3.3a). Cross-linked DNA, DNA with multiple repeat structure and contaminating material will not be digested. The time courses of S1 redigestion are illustrated in figure 3.4. These show that 20% of R. vannielii IR DNA and 50% of E. coli IR DNA is resistant to redigestion. This probably represents that fraction of the precipitable counts not attributable to inverted repeat sequence DNA. This allows revised estimates of the IR DNA abundance in the R. vannielii and E. coli genomes of 7% and 3.5% respectively.

Figure 3.3 Time course of nuclease S1 digestion of heat-denatured, rapidly renatured R.vannielii (closed circles) and E.coli K12 (open circles) DNA. In each case initial acid-precipitable activity was >10^4 cpm.



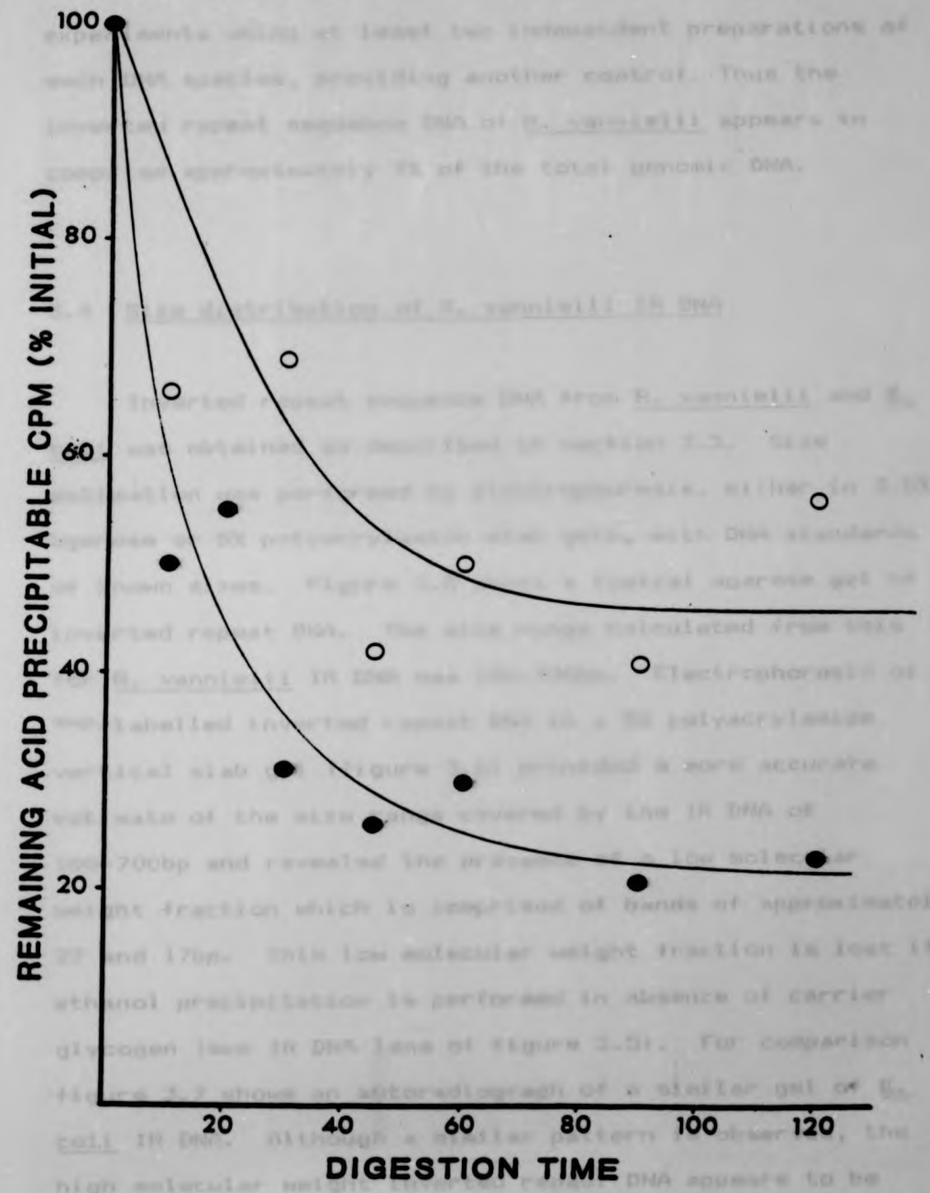


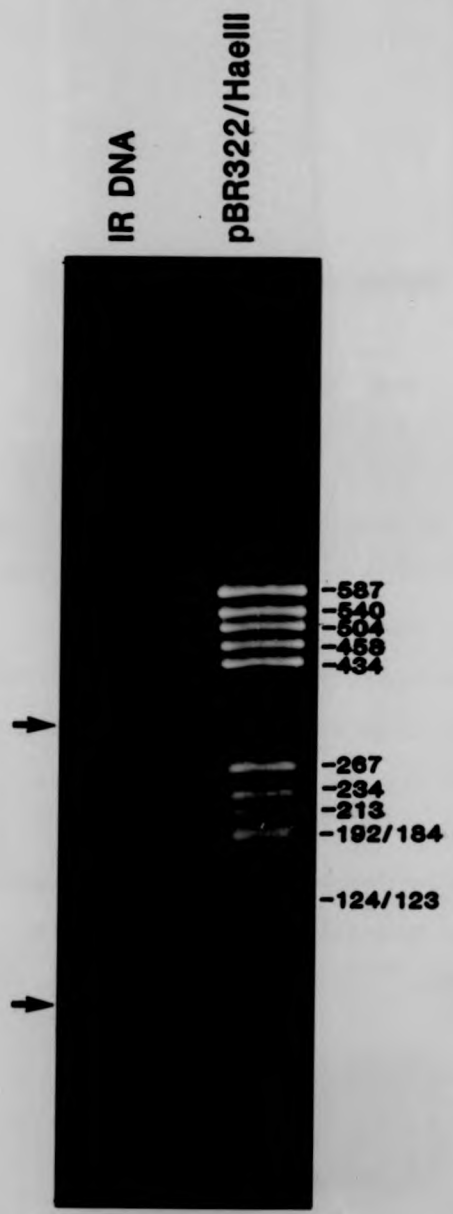
Figure 3.4 Time course of nuclease S1 digestion of heat-denatured, rapidly renatured S1-stable material from an initial limit digest (i.e. IR DNA) of R.vannielii (closed circles) and E.coli K12 (open circles) DNA. In each case initial acid-precipitable activity was $>10^3$ cpm.

The results presented above are averages from several experiments using at least two independent preparations of each DNA species, providing another control. Thus the inverted repeat sequence DNA of R. vannielii appears to comprise approximately 7% of the total genomic DNA.

3.4 Size distribution of R. vannielii IR DNA

Inverted repeat sequence DNA from R. vannielii and E. coli was obtained as described in section 3.3. Size estimation was performed by electrophoresis, either in 2.5% agarose or 5% polyacrylamide slab gels, with DNA standards of known sizes. Figure 3.5 shows a typical agarose gel of inverted repeat DNA. The size range calculated from this for R. vannielii IR DNA was 100-300bp. Electrophoresis of ^{32}P-labelled inverted repeat DNA in a 5% polyacrylamide vertical slab gel (figure 3.6) provided a more accurate estimate of the size range covered by the IR DNA of 100-700bp and revealed the presence of a low molecular weight fraction which is comprised of bands of approximately 27 and 17bp. This low molecular weight fraction is lost if ethanol precipitation is performed in absence of carrier glycogen (see IR DNA lane of figure 3.5). For comparison figure 3.7 shows an autoradiograph of a similar gel of E. coli IR DNA. Although a similar pattern is observed, the high molecular weight inverted repeat DNA appears to be present in reduced quantities relative to the low molecular weight fraction when compared with R. vannielii. It is also

Figure 3.5 EtBr stained 2.5% agarose gel of *R. vannielii* inverted repeat sequence DNA (IR DNA; 1µg) and *Hae*III digested plasmid pBR322 (5µg) as size standards, fragment sizes as indicated. The approximate size limits of the IR DNA are indicated by the arrows.


IR DNA
pBR322/HaeIII
-587
-540
-504
-458
-434
-267
-234
-213
-192/184
-124/123

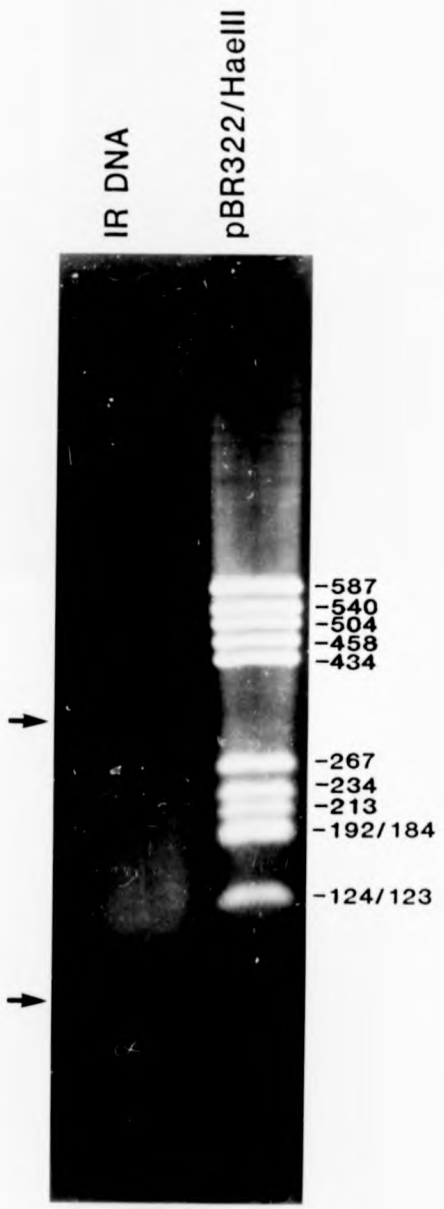
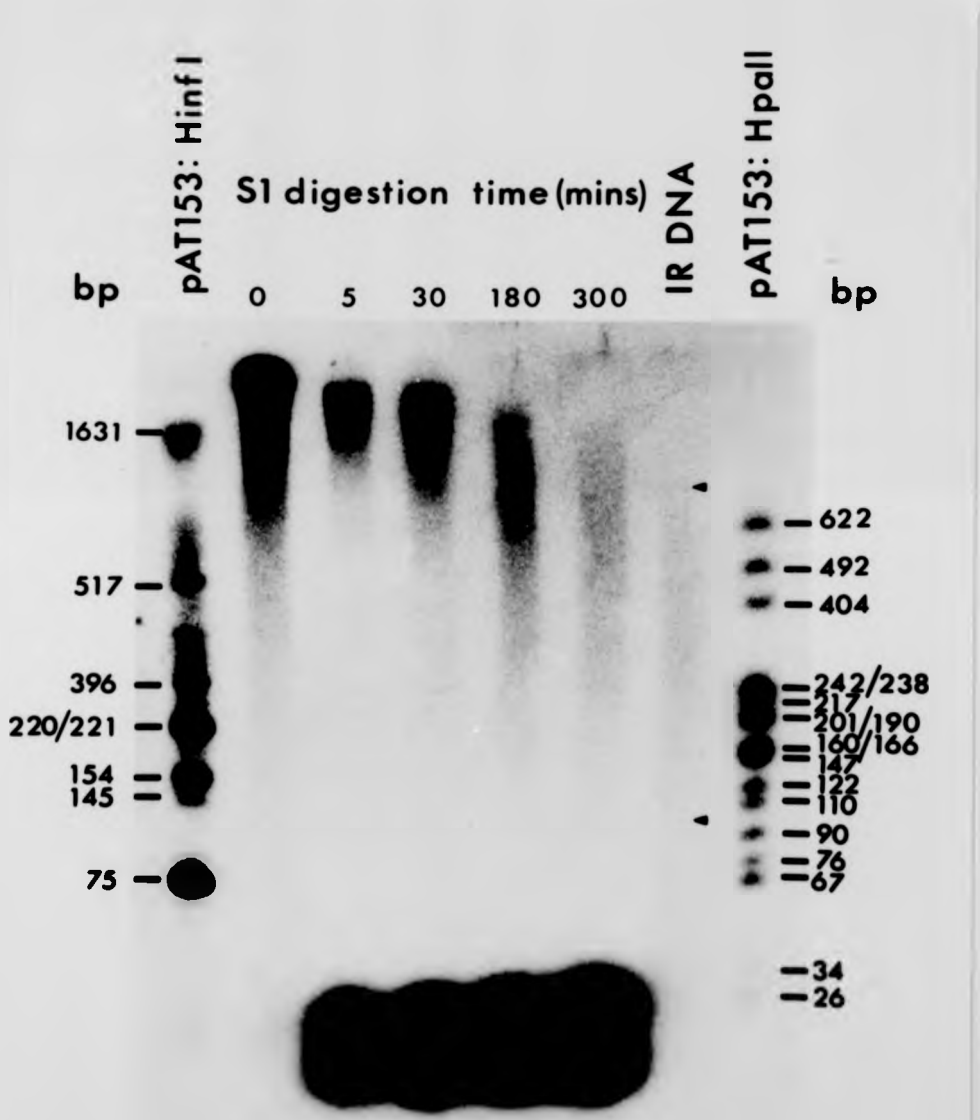
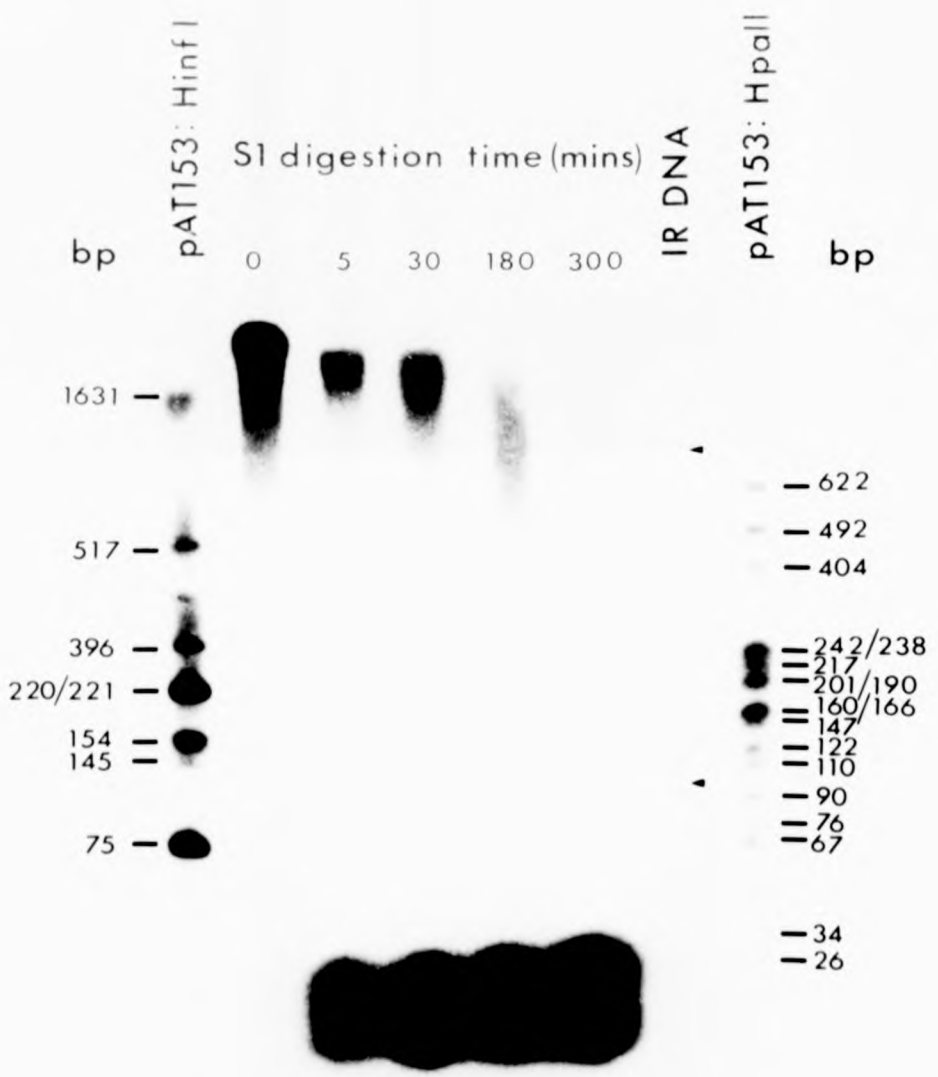

IR DNA
pBR322/HaeIII
-587
-540
-504
-458
-434
-267
-234
-213
-192/184
-124/123

Figure 3.6 Autoradiograph of a 5% polyacrylamide gel of samples from a nuclease S1 digestion of R. vannielii DNA.

IR DNA: Ethanol precipitated material from a limit digestion (360 minutes). The approximate size limits of the IR DNA are indicated by the arrowheads.

Size standards: plasmid pAT153 digested with HinfI or HpaII, end-labelled with ^{32}P (fragment sizes as indicated).


pAT153: HinfI
S1 digestion time (mins)
IR DNA
pAT153: HpaII
bp
0
5
30
180
300
bp
1631
517
396
220/221
154
145
75
622
492
404
242/238
217
201/190
160/166
147
122
110
90
76
67
34
26


pAT153: HinfI
S1 digestion time (mins)
IR DNA
pAT153: HpaII
bp
0
5
30
180
300
bp
1631
517
396
220/221
154
145
75
622
492
404
242/238
217
201/190
160/166
147
122
110
90
76
67
34
26

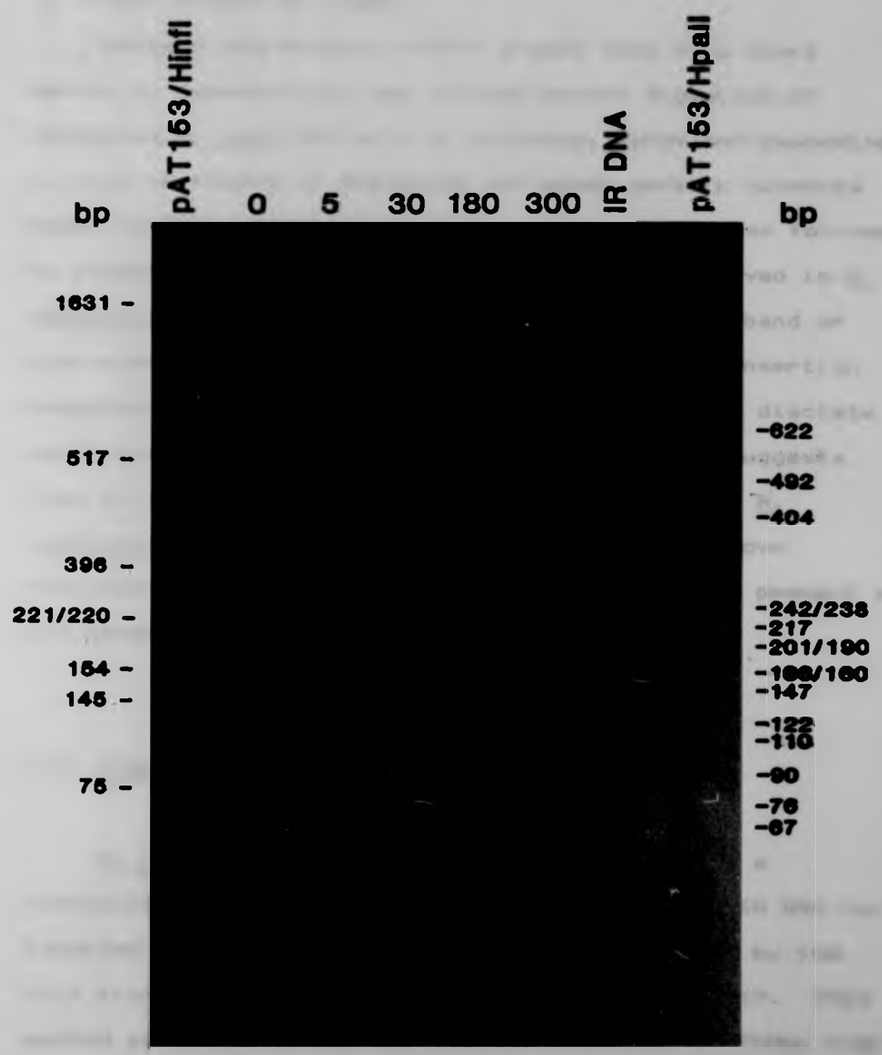


Figure 3.7 Autoradiograph of a 5% polyacrylamide gel of samples from a nuclease S1 digestion of *E. coli* DNA.

IR DNA: Ethanol precipitated material from a limit digestion (360 minutes).

Size standards: plasmid pAT153 digested with *Hin*fI or *Hpa*II, end-labelled with ^{32}P (fragment sizes as indicated).

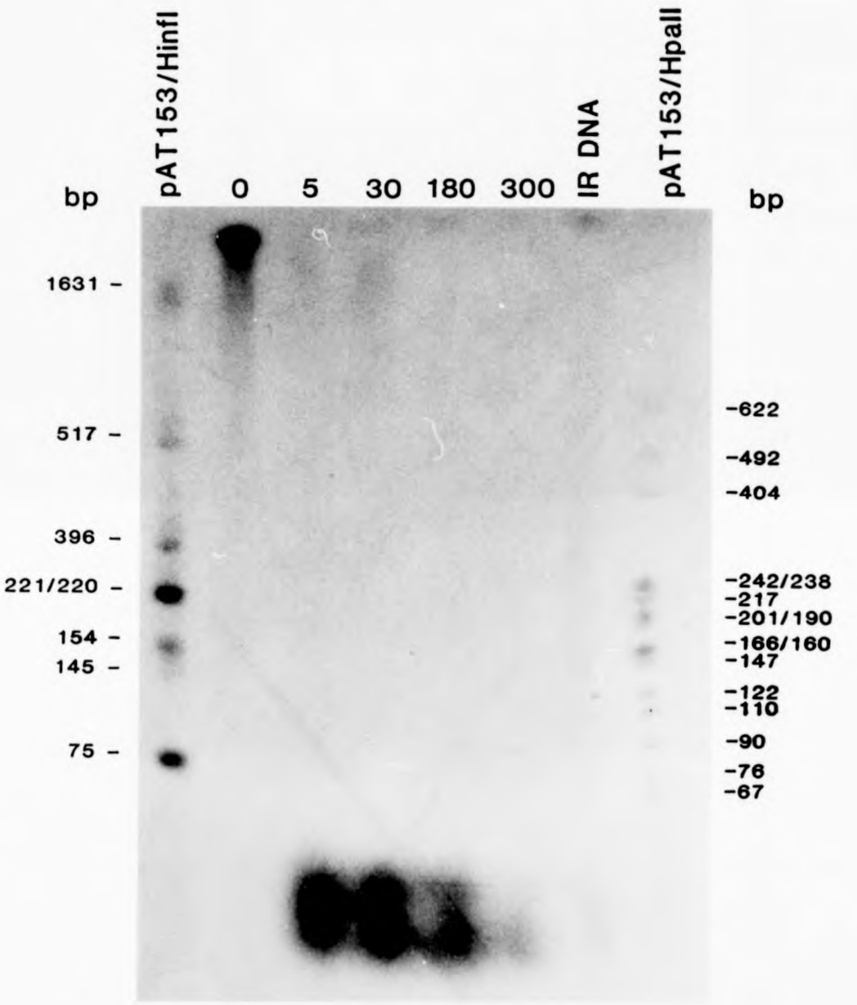


Figure 3.7 Autoradiograph of a 5% polyacrylamide gel of samples from a nuclease S1 digestion of *E. coli* DNA.

IR DNA: Ethanol precipitated material from a limit digestion (360 minutes).

Size standards: plasmid pAT153 digested with HinfI or HpaII, end labelled with ^{32}P (fragment sizes as indicated).

apparent that the low molecular weight fraction is degraded at longer digestion times.

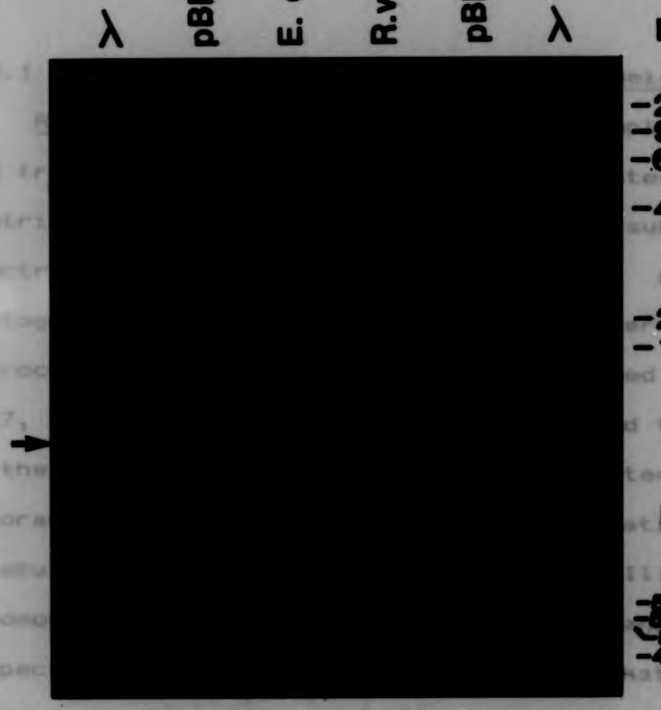
Ohtsubo and Ohtsubo (1977) showed that if a short period of renaturation was allowed before digestion of denatured E. coli DNA with S1 nuclease, bands corresponding in size to Insertion Sequences and other genetic elements could be observed on agarose gels. This method was followed to determine whether such elements could be observed in R. vannielii DNA. Figure 3.8 shows that although a band of approximately 1200bp (possibly corresponding to Insertion Sequence IS2) can be seen in the E. coli lane, no discrete bands are found in the R. vannielii lane. This suggests that no such repeated elements are present in R. vannielii DNA and that the sequences described above represent all of the inverted repeat sequence DNA present in the genome.

3.5 Hybridization of IR DNA to total genomic DNA

R. vannielii inverted repeat DNA was used as a radioactive probe in hybridization experiments. IR DNA was labelled to high specific activity with α^{32}P-dGTP by the nick translation protocol described in section 2.19. This method proved efficient for labelling of the 100-700bp high molecular weight IR DNA fraction but was ineffective in labelling the low molecular weight (17 & 27bp) fragments. In the course of labelling it was discovered that the high and low molecular weight IR DNA fractions could be separated by gel filtration on a 2ml column of Sephadex G-50

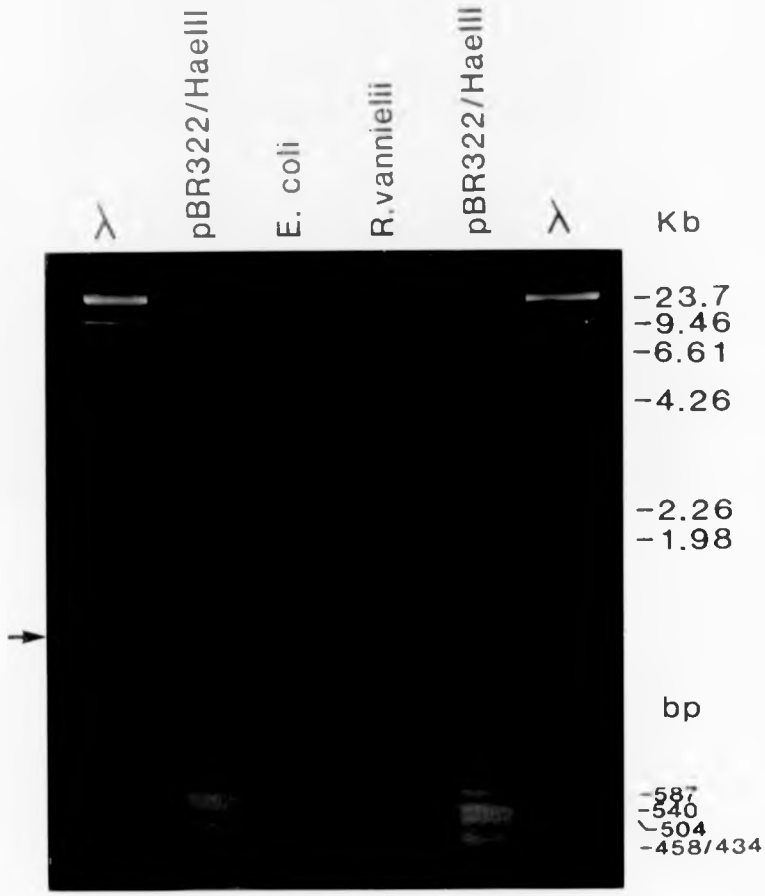
Figure 3.8 EtBr stained 1.5% agarose gel of nuclease S1 digested heat-denatured, partially renatured (30s, 68°C) DNA from *R. vannielii* and *E. coli* (10µg each digested). Arrow indicates a faint band in the *E. coli* lane.
Size standards: *Hin*dIII digested λ (1µg) and *Hae*III digested pBR322 DNA (3µg), fragment sizes as indicated.

illustrated in figure 3.4). This allowed the low molecular weight IR DNA fraction to be isolated and used as an independent probe. Low molecular weight IR DNA was labelled to high specific activity with ^{32}P-ATP by the end-labelling protocol described in section 2.20. Thus both IR DNA fractions could be used independently as probes in hybridization experiments.



3.3.1 [illegible] weight IR DNA

[illegible] cycle cells and [illegible] with the restriction [illegible] subjected to electrophoresis. After [illegible] to nitrocellulose [illegible] in section 2.17, [illegible] to the filters by the [illegible] filters autoradiography [illegible] of high molecular [illegible] digested chromosomal [illegible] respectively [illegible] that the high molecular weight fraction of R. vannielii IR DNA shares homology with many bands in both the EcoRI and HindIII restrictions, suggesting that this fraction consists of many species which are dispersed throughout the genome and do not show clustering. Careful observation of the hybridization patterns reveals that there are no significant differences between the banding patterns of the different DNA samples used. These results suggest that cell cycle-associated DNA rearrangements involving IR DNA sequences are unlikely but

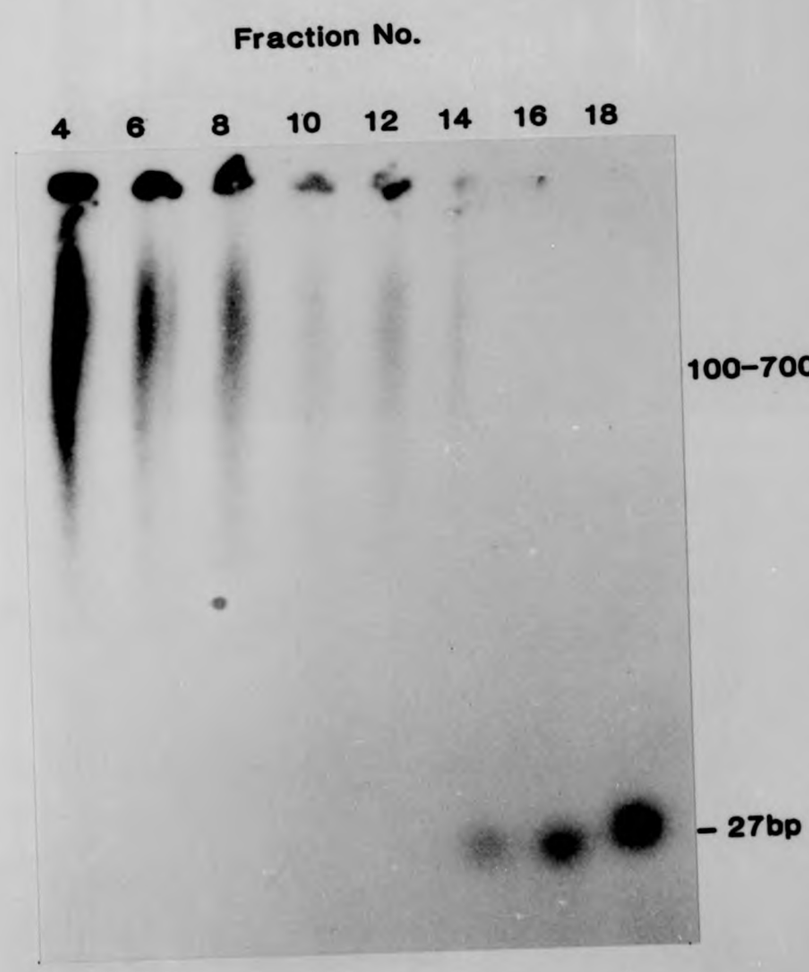

λ
pBR322/HaeIII
E. coli
R.vannielii
pBR322/HaeIII
λ
Kb
-23.7
-9.46
-6.61
-4.26
-2.26
-1.98
bp
-587
-540
-504
-458/434

(illustrated in figure 3.9). This allowed the low molecular weight IR DNA fraction to be isolated and used as an independent probe. Low molecular weight IR DNA was labelled to high specific activity with γ^{32}P-ATP by the end-labelling protocol described in section 2.20. Thus both IR DNA fractions could be used independently as probes in hybridization experiments.

3.5.1 Hybridization with high molecular weight IR DNA

R. vannielii chromosomal DNA from complex cycle cells and from 0h and 6h swarmer cells was digested with the restriction enzymes *Eco*RI and *Hind*III and subjected to electrophoresis in 0.7% agarose slab gels. After photography the DNA in the gels was transferred to nitrocellulose membrane filters as described in section 2.17, ^{32}P-labelled probe DNA was hybridized to the filters by the method in section 2.18, and the filters autoradiographed. The results of hybridization of high molecular weight IR DNA to *Eco* RI and *Hind*III digested chromosomal DNA are shown in figures 3.10 and 3.11 respectively. These figures demonstrate that the high molecular weight fraction of *R. vannielii* IR DNA shares homology with many bands in both the *Eco*RI and *Hind*III restrictions, suggesting that this fraction consists of many species which are dispersed throughout the genome and do not show clustering. Careful observation of the hybridization patterns reveals that there are no significant differences between the banding patterns of the different DNA samples used. These results suggest that cell cycle-associated DNA rearrangements involving IR DNA sequences are unlikely but

Figure 3.9 Autoradiograph of a 5% polyacrylamide gel of fractions from a sephadex G-50 column loaded with R. vannielii total IR DNA.



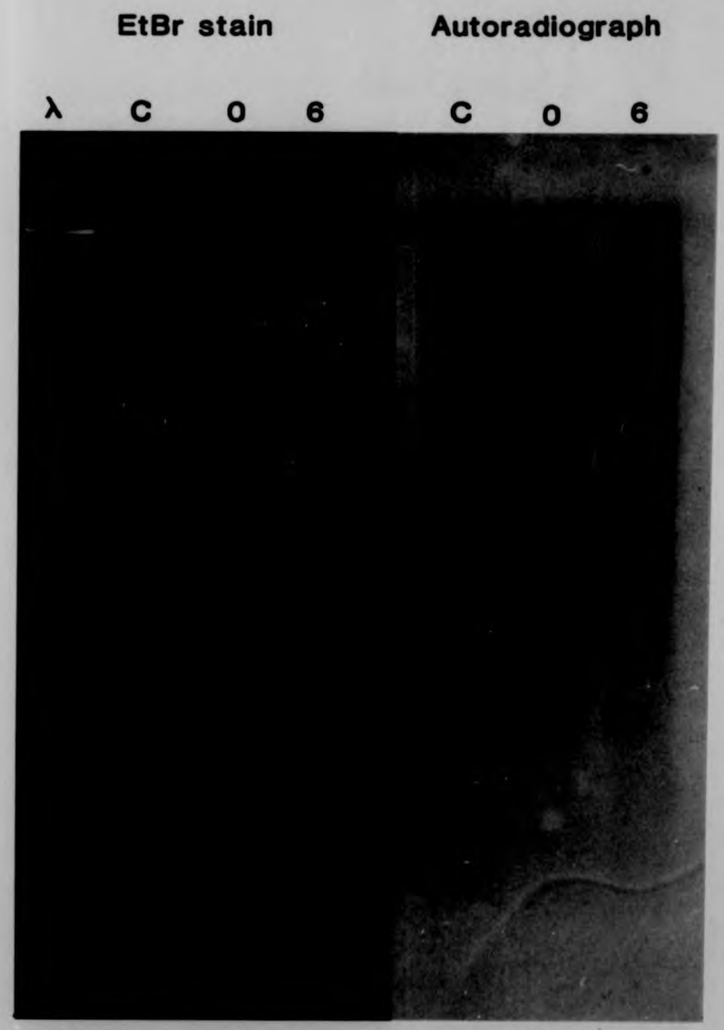


Figure 3.10 EtBr stained 0.7% agarose gel of EcoR1 digested complex cycle (C), 0h swarmer (0) and 6h swarmer (6) DNA (5μg each) with HindIII digested λ standards (1μg) and autoradiograph of the corresponding hybridization with nick-translated R. vannielii 100-700 bp IR DNA probe. Input activity >10^4 cpm.

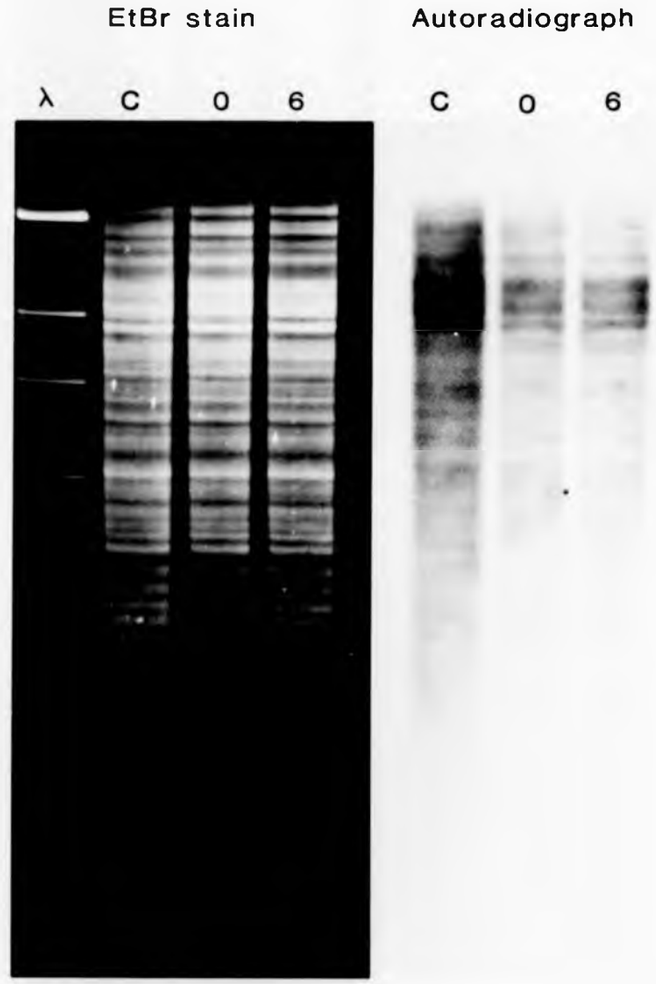


Figure 3.10 EtBr stained 0.7% agarose gel of EcoR1 digested complex cycle (C), 0h swarmer (0) and 6h swarmer (6) DNA (5μg each) with HindIII digested λ standards (1μg) and autoradiograph of the corresponding hybridization with nick-translated *R. vannielii* 100-700 bp IR DNA probe. Input activity ~10^6 cpm.

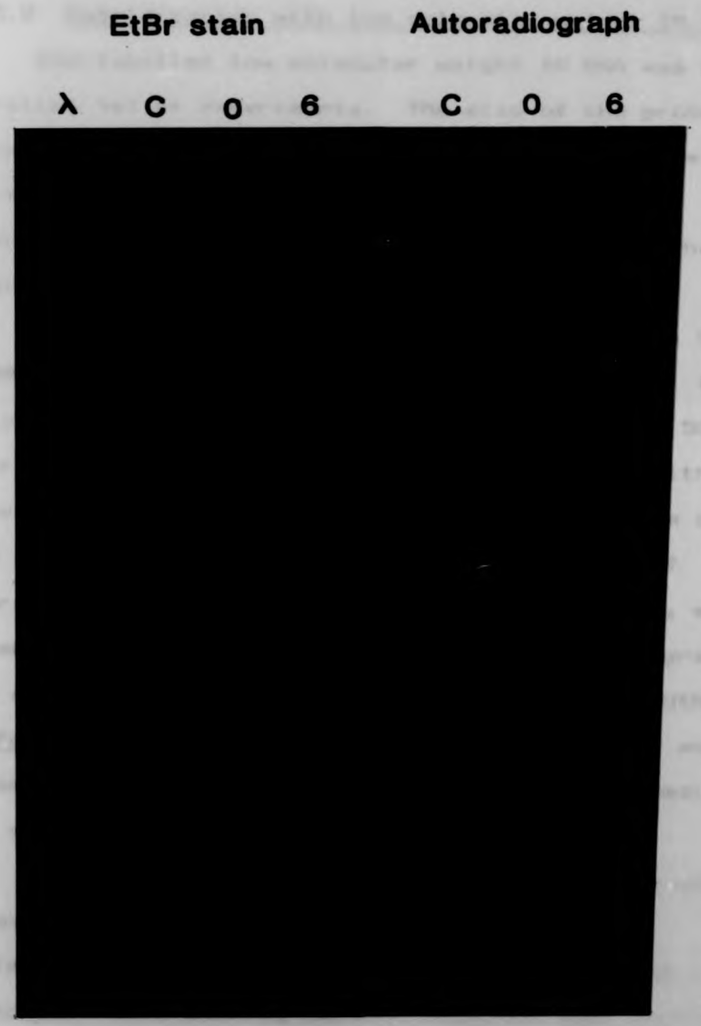


Figure 3.11 EtBr stained 0.7% agarose gel of HindIII digested complex cycle (C), 0h swarmer (O) and 6h swarmer (6) DNA (5μg each) with HindIII digested λ standards (1μg) and autoradiograph of the corresponding hybridization with nick-translated R. vannielii 100-700 bp IR DNA probe. Input activity >10^4 cpm.

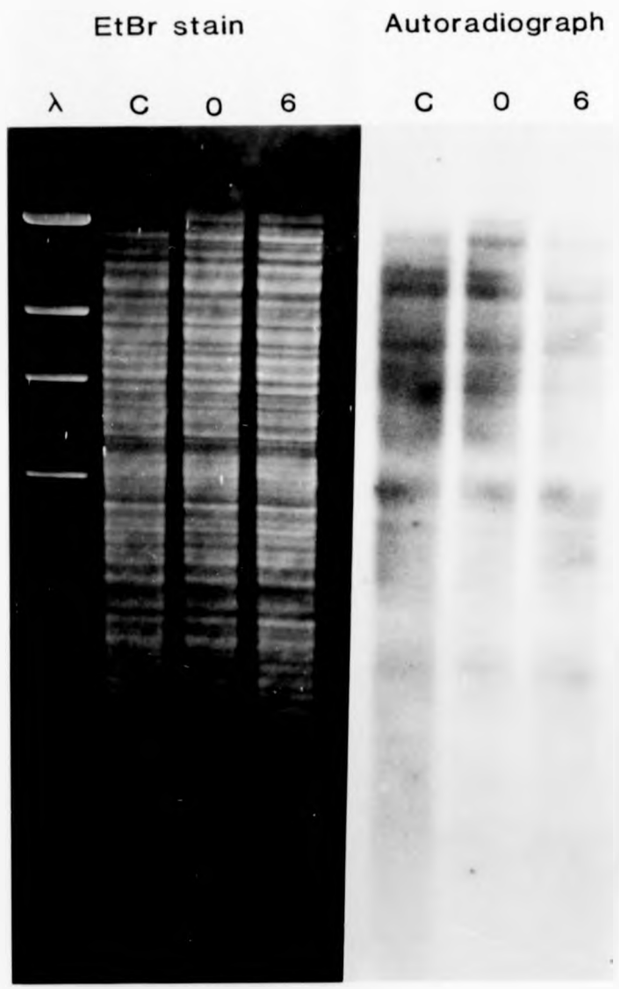


Figure 3.11 EtBr stained 0.7% agarose gel of HindIII digested complex cycle (C), 0h swarmer (0) and 6h swarmer (6) DNA (5μg each) with HindIII digested λ standards (1μg) and autoradiograph of the corresponding hybridization with nick-translated R. vannielii 100-700 bp IR DNA probe. Input activity >10^6 cpm.

further work would be required to substantiate this suggestion.

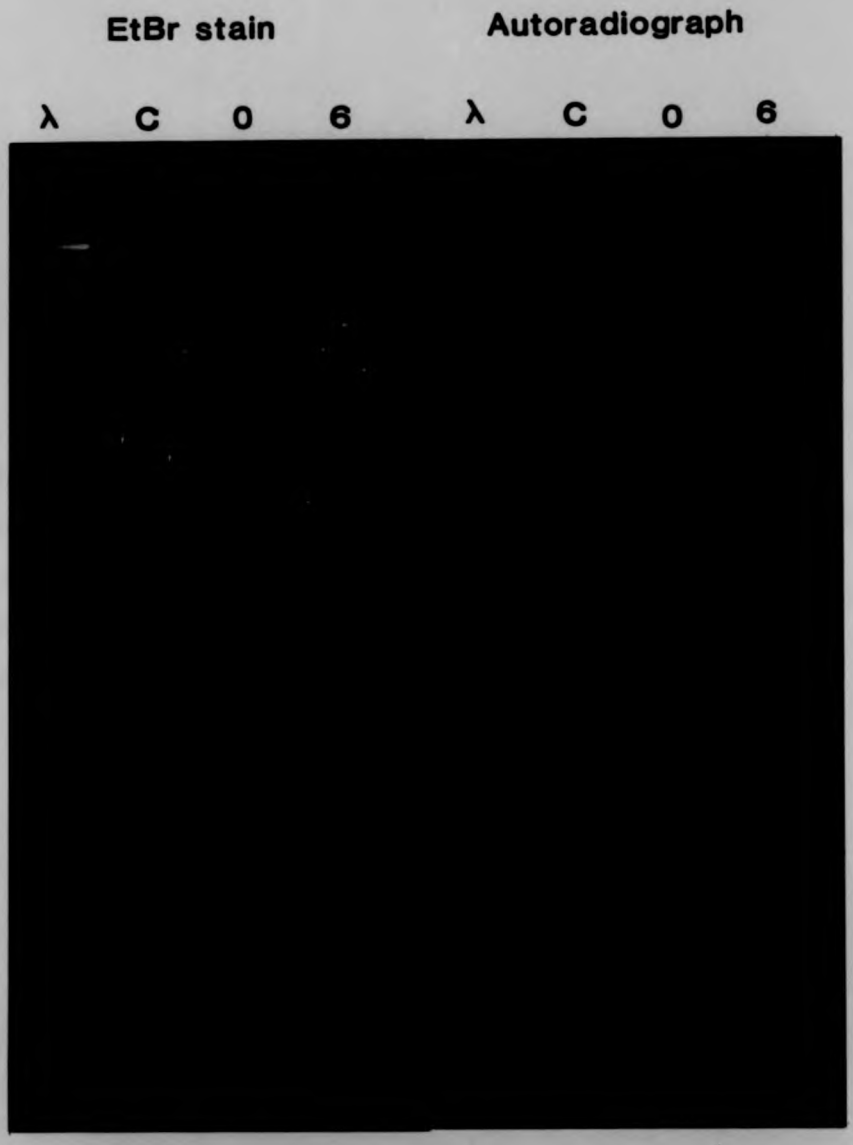
3.5.2 Hybridization with low molecular weight IR DNA

End-labelled low molecular weight IR DNA was used in a parallel set of experiments. The size of the probe molecules however, dictated that a different, less stringent, set of conditions should be used for hybridization. The method used was essentially that described by Wallace et al. (1981) as follows.

Filters were prehybridized for 1h at 37°C in 0.9M NaCl, 0.09M Tris/HCl pH 7.5, 0.006M Na_2EDTA, 0.1% (w/v) ficoll, polyvinylpyrrolidone and BSA, 0.5% (w/v) SDS and 50μM ATP. Hybridization was performed in the same buffer with 10% (w/v) dextran sulphate and approximately 10^4 cpm of probe DNA in sealed polythene bags. After about 20h of hybridization at 37°C the filters were retrieved, washed extensively in 6xSSC at 0°C, dried and autoradiographed. Low molecular weight IR DNA was hybridized to Southern blots of EcoRI and HindIII digestions of complex cycle and 0h and 6h swarmer cell DNA. The stained gels and autoradiographs are shown in figures 3.12 and 3.13.

As can be seen, low molecular weight IR DNA hybridizes to many bands within each digest and no cell cycle-associated changes in hybridization pattern can be detected. This banding pattern suggests that either the low molecular weight IR DNA fraction consists of many species, or of a few species which are repeated throughout the genome. An alternative explanation is that the low stringency allowed hybridization to sequences which were not

Figure 3.12 EtBr stained 0.7% agarose gel of EcoR1 digested complex cycle (C), 0h swarmer (0) and 6h swarmer (6) DNA (5μg each) with HindIII digested λ standards (1μg) and autoradiograph of the corresponding hybridization with end-labelled R. vannielii 17/27 bp IR DNA probe. Input activity >10^6 cpm.


EtBr stain
Autoradiograph
λ C 0 6
λ C 0 6

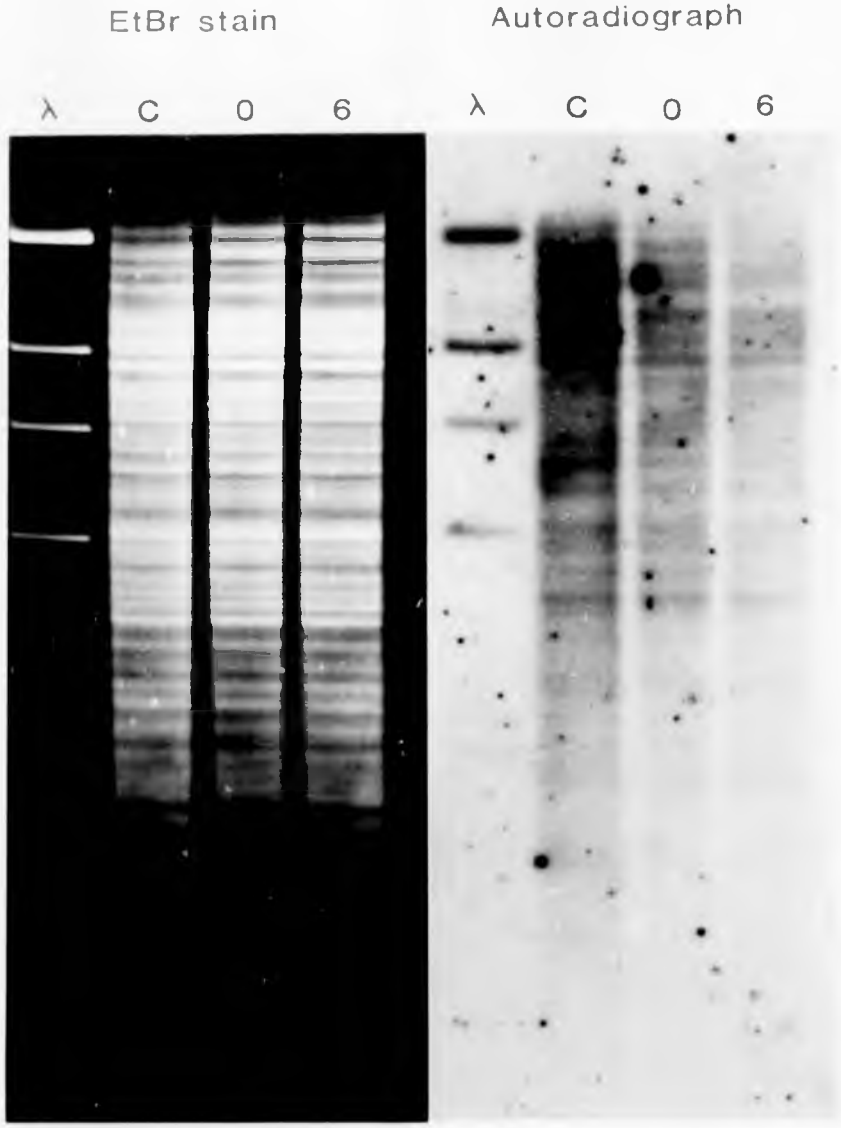
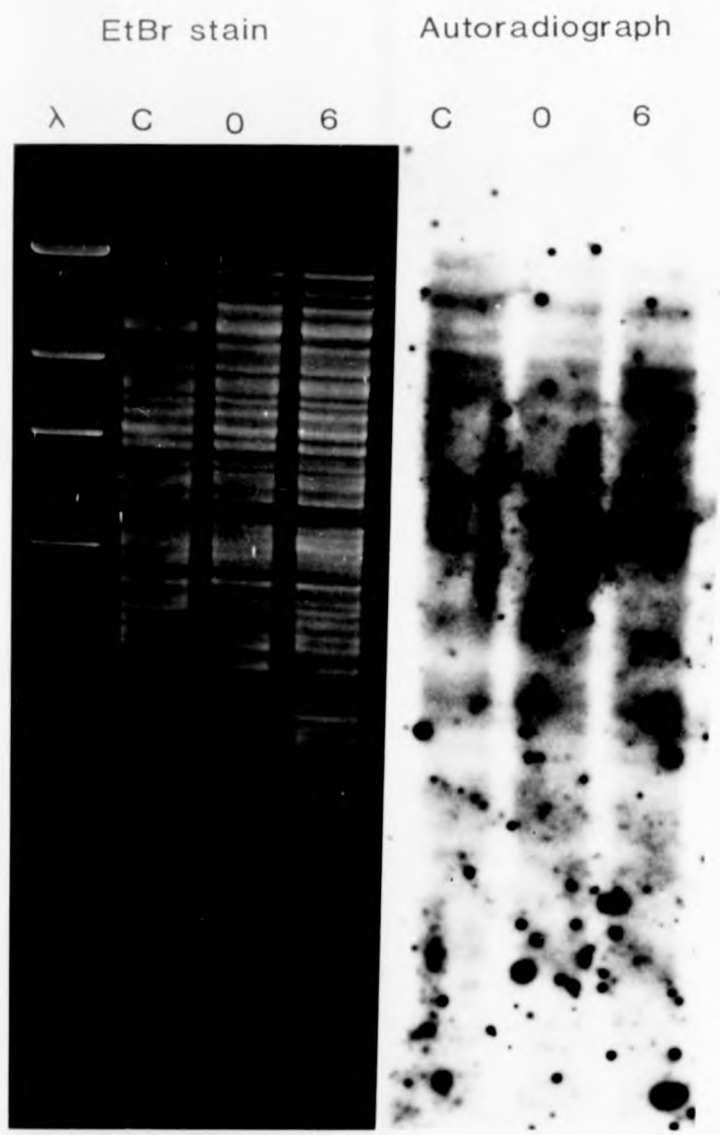

EtBr stain
Autoradiograph
λ C 0 6
λ C 0 6

Figure 3.13 EtBr stained 0.7% agarose gel of HindIII digested complex cycle (C), 0h swarmer (0) and 6h swarmer (6) DNA (5μg each) with HindIII digested λ standards (1μg) and autoradiograph of the corresponding hybridization with end-labelled R. vannielii 17/27 bp IR DNA probe. Input activity >10^6 cpm.


EtBr stain
Autoradiograph
λ C 0 6 C 0 6


EtBr stain
Autoradiograph
λ C 0 6
C 0 6

completely homologous. This explanation is supported by the observed hybridization to phage λ DNA in figure 3.12. To resolve this question and to determine if there was any homology between the low and high molecular weight IR DNA components, low molecular weight IR DNA was hybridized to a Southern blot of a 3% agarose slab gel containing high molecular weight IR DNA, HaeIII digested complex cycle DNA and HaeIII digested pBR322 DNA. The autoradiograph in figure 3.14 illustrates a number of points:

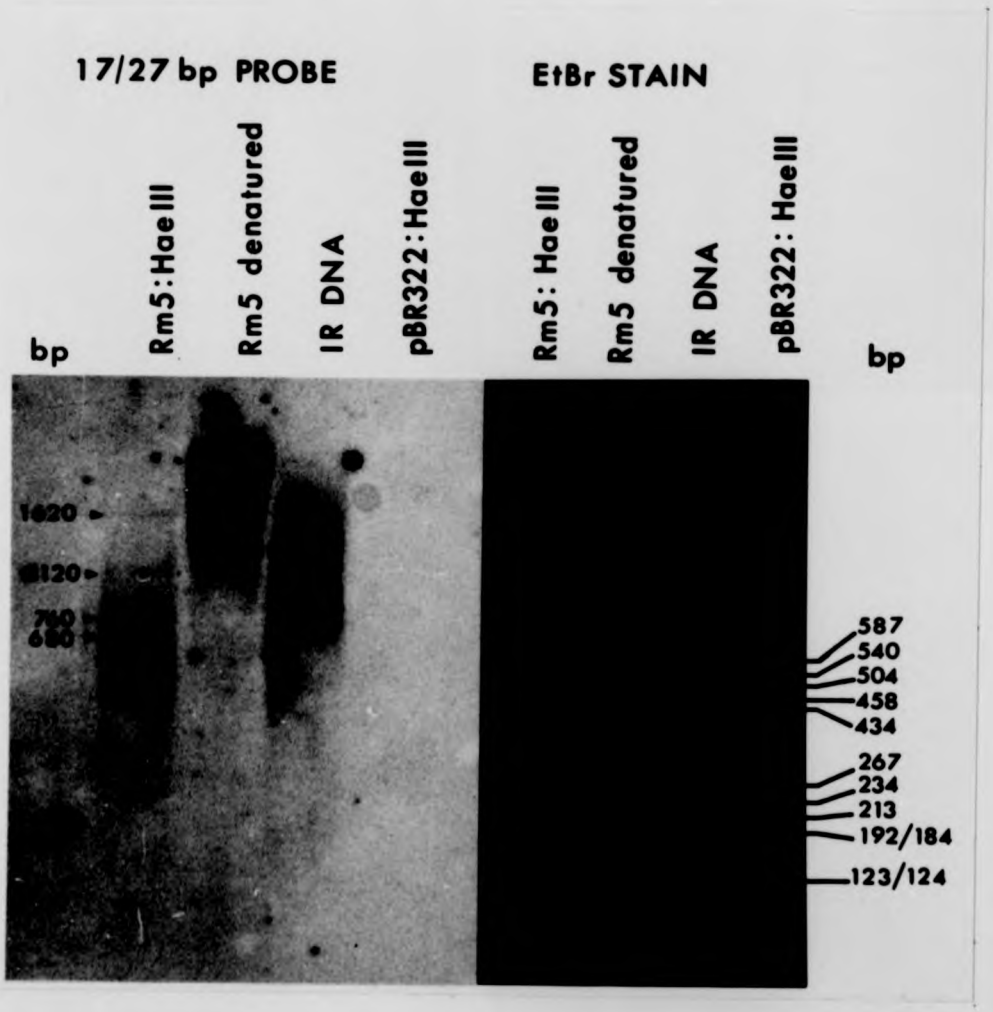
1. Hybridization to HaeIII digested chromosomal DNA is limited to a small number of major bands - 4 can be seen on close examination.
2. Hybridization to high molecular weight IR DNA appears to be stronger in the higher molecular weight region.
3. No hybridization to pBR322 DNA is observed suggesting that the stringency of hybridization is high enough to eliminate non-specific interactions.

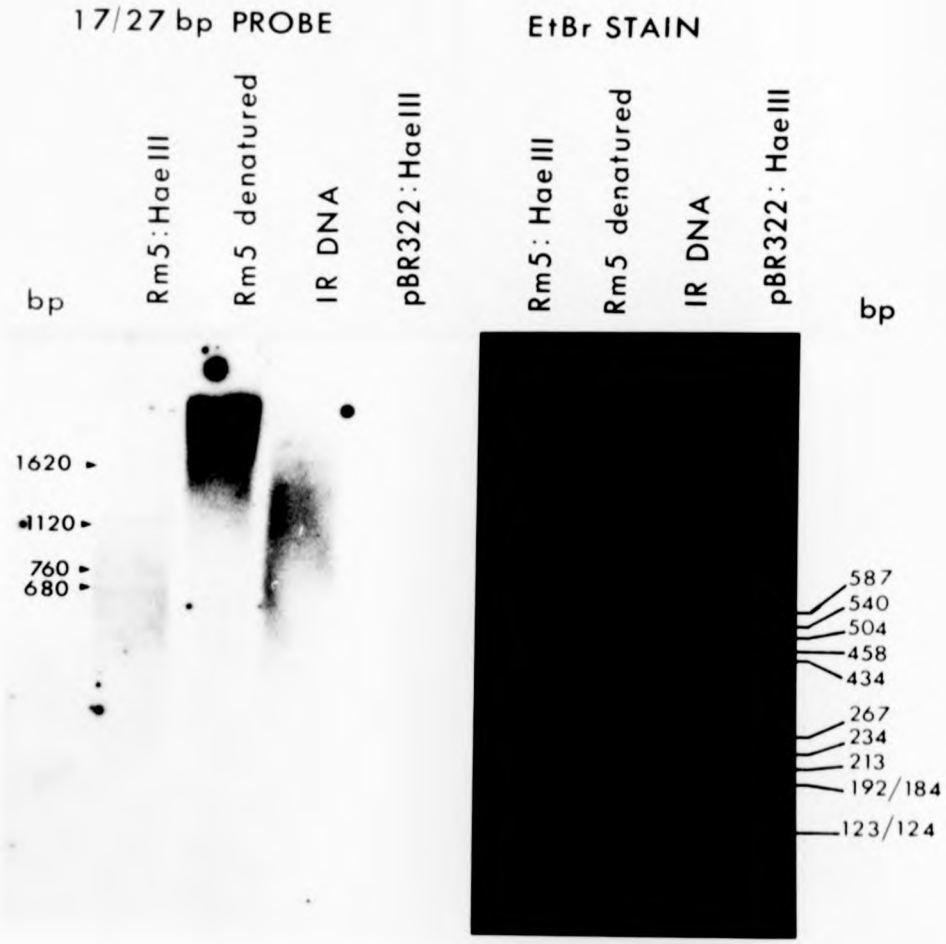
The differences in hybridization of low molecular weight IR DNA to the EcoRI, HindIII and HaeIII digestions may reflect the organisation of these IR DNA sequences into repetitive blocks which are observed as the individual bands in the HaeIII hybridization, but which are not revealed by digestion with EcoRI or HindIII.

3.6 Interaction of IR DNA with R. vannielii protein

If inverted repeat sequences have any role in the cell cycle of R. vannielii, it will probably be mediated by proteins which bind to certain inverted repeat sequences in

Figure 3.14 EtBr stained 3% agarose gel of HaeIII digested R.vannielii DNA (RM5/HaeIII; 2µg), heat-denatured R. vannielii DNA (RM5 denatured; 1µg) and R. vannielii IR DNA (IR DNA; 2µg) with HaeIII digested pBR322 standards (2µg; fragment sizes as figure 3.5) and autoradiograph of the corresponding hybridization with end-labelled R. vannielii 17/27 bp IR DNA probe. Input activity $>10^{8}$ cpm.


17/27 bp PROBE
EtBr STAIN
bp
Rm5:HaeIII
Rm5 denatured
IR DNA
pBR322:HaeIII
Rm5: HaeIII
Rm5 denatured
IR DNA
pBR322: HaeIII
bp
1020
120
587
540
504
458
434
267
234
213
192/184
123/124


17/27 bp PROBE
EtBr STAIN
bp
Rm5:HaeIII
Rm5 denatured
IR DNA
pBR322:HaeIII
Rm5: HaeIII
Rm5 denatured
IR DNA
pBR322: HaeIII
bp
1620
1120
760
680
587
540
504
458
434
267
234
213
192/184
123/124

a sequence-specific manner. To demonstrate such sequence-specific DNA-protein interactions a competition binding assay system was used. In these assays radioactively labelled substrate DNA was allowed to bind to the protein to be tested in the presence of various amounts of non-homologous competing DNA. The DNA-protein complexes formed were collected by filtration on nitrocellulose filters which were subsequently assayed for radioactive DNA by scintillation counting. Thus the specificity of the interaction between the radioactive DNA and the protein used can be estimated from the decay in protein-bound counts in the presence of increasing amounts of competing DNA. The protocol used in these experiments is based on the methods of Lye & Birge (1981) and Strauss et al. (1981) and is described below:

1. Binding reaction

DNA-protein binding mixtures contained approximately $2x10^4$ cpm (1ng) of ^{32}P-labelled 100-700bp IR DNA, 50µg of R. vannielii soluble protein prepared as described in section 2.21, and up to 5µg of competing calf thymus DNA (sheared to an average length of 300bp by sonication) in binding buffer (10mM Tris/HCl pH 7.4, 50mM NaCl, 1mM Na_2EDTA, 1mM 2-mercaptoethanol). Assays were prepared on ice and were incubated for 30 minutes at 4°C after addition of protein extract.

2. Preparation of filters

Nitrocellulose filter discs (Millipore HA, 0.45µm) were prepared by soaking in 0.4M KOH for 20 minutes, neutralisation in 500mM Tris/HCl pH 7.4 and thorough washing in binding buffer. Filters were stored in binding buffer

before use, usually on the same day.

3. Filtration of binding assays

DNA-protein binding mixtures were passed through prepared filters under vacuum at a flow rate of about 5ml/minute and were washed with 2x1ml of binding buffer. Filters were then dried and bound radioactivity determined by scintillation counting.

Three groups of experiments were performed using such assays:

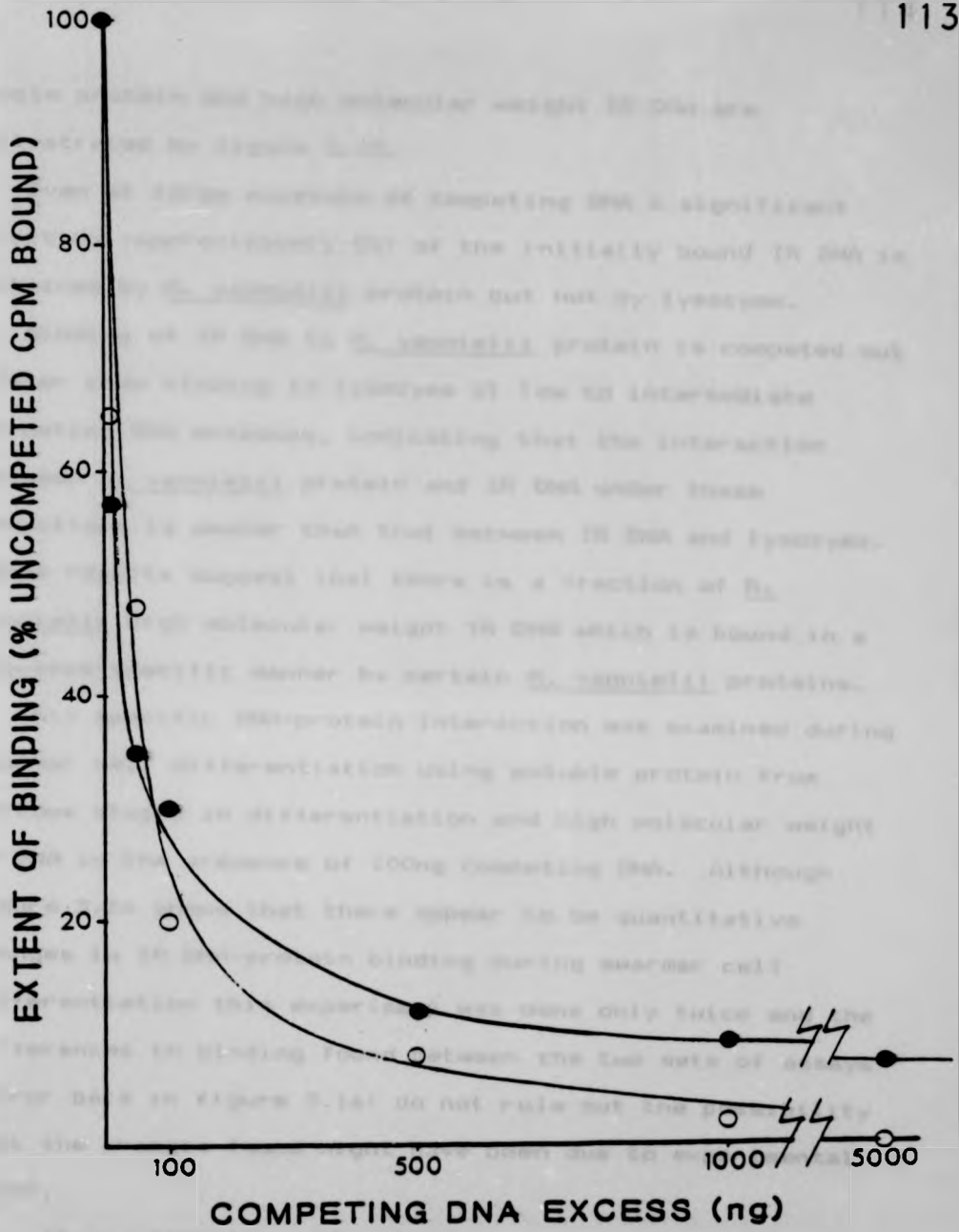
a. Binding of high molecular weight IR DNA to complex cycle protein.

b. Binding of high molecular weight IR DNA to protein from differentiating swarmer cells.

c. Binding of low molecular weight IR DNA to complex cycle protein

a. As described above, 20,000 cpm (1ng) of ^{32}P-labelled high molecular weight IR DNA and 50µg of complex cycle protein were incubated together in the presence of up to 5µg of sheared calf thymus DNA (equivalent to 5000x excess by weight). For each experiment the counts retained on the filters were expressed as a percentage of the counts bound in absence of competing DNA. Figure 3.15 shows the combined results of four experiments using competing DNA excesses of up to x5000. As an indication of the effects of competition on non-specific DNA-protein binding, a parallel set of experiments was performed using 1µg of lysozyme instead of R. vannielii protein. Lysozyme was used because it is known to bind DNA strongly and non-specifically (Lye & Birge, 1981). Two features of the interaction between complex

Figure 3.15 Competition filter-binding assay of the interaction of 50µg *R. vannielii* complex cycle soluble protein (closed circles) or 1µg lysozyme (open circles) with ~1ng ^{32}P-labelled *R. vannielii* 100-700 bp IR DNA, in the presence of increasing amounts of competing DNA. Input activity was ~10^4 cpm/assay and counts bound in the absence of competing DNA were 20-30% of this for both lysozyme and *R. vannielii* protein.


100
80
60
40
20
EXTENT OF BINDING (% UNCOMPETED CPM BOUND)
100
500
1000
5000
COMPETING DNA EXCESS (ng)

cycle protein and high molecular weight IR DNA are illustrated by figure 3.15.

1. Even at large excesses of competing DNA a significant fraction (approximately 8%) of the initially bound IR DNA is retained by R. vannielii protein but not by lysozyme.

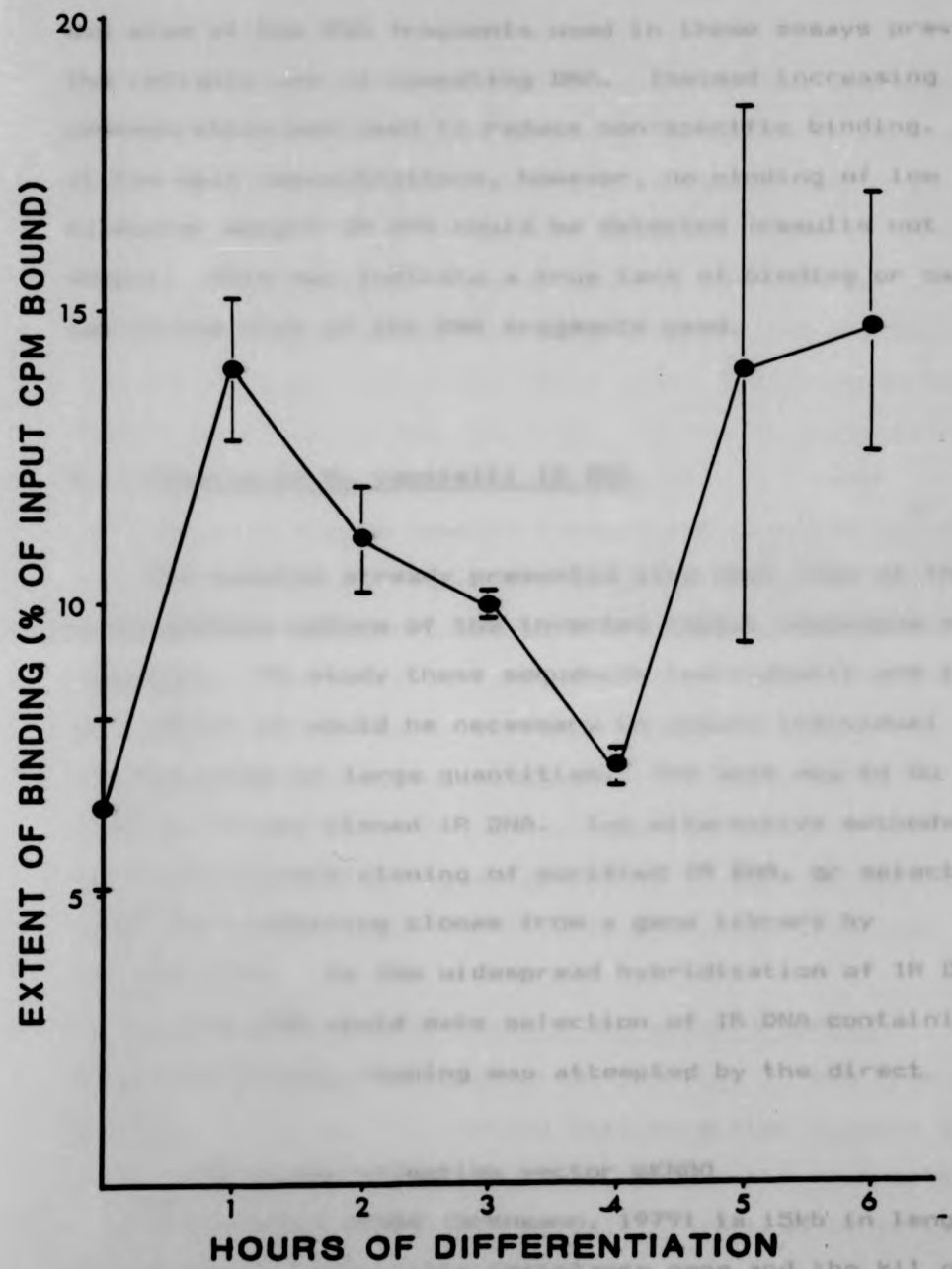
2. Binding of IR DNA to R. vannielii protein is competed out faster than binding to lysozyme at low to intermediate competing DNA excesses, indicating that the interaction between R. vannielii protein and IR DNA under these conditions is weaker than that between IR DNA and lysozyme. These results suggest that there is a fraction of R. vannielii high molecular weight IR DNA which is bound in a sequence-specific manner by certain R. vannielii proteins.

b. This specific DNA-protein interaction was examined during swarmer cell differentiation using soluble protein from various stages in differentiation and high molecular weight IR DNA in the presence of 100ng competing DNA. Although Figure 3.16 shows that there appear to be quantitative changes in IR DNA-protein binding during swarmer cell differentiation this experiment was done only twice and the differences in binding found between the two sets of assays (error bars in figure 3.16) do not rule out the possibility that the changes found might have been due to experimental error.

It was also found that the binding activity of these protein extracts was highly temperature-labile, being reduced to undectable levels after incubation for one hour at room temperature (results not shown).

c. Specific binding of the low molecular weight IR DNA fraction was also investigated, but the very small amounts

Figure 3.16 Filter-binding assay of the interaction between soluble protein from differentiating *R. vannielii* swarmer cells and ^{32}P-labelled *R. vannielii* IR DNA in the presence of 100x excess competing DNA. Input activity was ~10^4 cpm/assay.


EXTENT OF BINDING (% OF INPUT CPM BOUND)
20
15
10
5
1
2
3
4
5
6
HOURS OF DIFFERENTIATION

and size of the DNA fragments used in these assays prevented the reliable use of competing DNA. Instead increasing salt concentration was used to reduce non-specific binding. Even at low salt concentrations, however, no binding of low molecular weight IR DNA could be detected (results not shown). This may indicate a true lack of binding or may be due to the size of the DNA fragments used.

3.7 Cloning of R. vannielii IR DNA

The results already presented give some idea of the heterogeneous nature of the inverted repeat sequences of *R. vannielii*. To study these sequences individually and in more detail it would be necessary to obtain individual IR DNA sequences in large quantities. The best way to do this would be to use cloned IR DNA. Two alternative methods were available: direct cloning of purified IR DNA, or selection of IR DNA containing clones from a gene library by hybridization. As the widespread hybridization of IR DNA to chromosomal DNA would make selection of IR DNA containing clones difficult, cloning was attempted by the direct method.

3.7.1 Use of the selection vector pKNBO

The plasmid pKNBO (Schumann, 1979) is 15kb in length and carries an ampicillin resistance gene and the *kil* gene from bacteriophage Mu. This gene is lethal to Mu-sensitive strains of *E. coli* but may be inactivated by insertion of DNA by blunt-end ligation into the unique *Hpa*I site. Thus on transformation of a Mu-sensitive host plasmids with

inserted DNA are positively selected for.

R. vannielii high molecular weight IR DNA and HpaI restricted pKN80 (0.5µg of each) were ligated in 50µl of ligation buffer (66mM Tris/HCl pH 7.6, 6.6mM $MgCl_2$, 10 mM Dithiothreitol (DTT), 0.4mM ATP) with 2.5 units of T4 DNA ligase (BRL). Ligation was allowed to continue for 3h at 18°C, then for a further 16h at 4°C before transformation of competent E.coli K12 strain HB101 cells according to the method described in section 2.22. Of the 46 ampicillin resistant colonies obtained approximately 50% were considered likely to contain recombinant plasmids by virtue of their increased frequency of transformation of HB101 (Native pKN80 has a transformation frequency for HB101 of less than 10^2 transformants/µg due to the action of the kil gene). The small number of recombinants obtained, the difficulty in unambiguously determining those which contained inserted IR DNA and the purification of such DNA away from the vector sequences led to another attempt to clone IR DNA sequences directly by a different method.

3.7.2 Use of molecular linkers and plasmid pBR322

To facilitate the removal of inserted IR DNA from a plasmid vector, it was decided that molecular linkers would be used to clone R. vannielii IR DNA into the unique PstI site of the 4.36 kb plasmid pBR322. Insertion of foreign DNA at this site inactivates the ampicillin (Ap) resistance gene leaving recombinant plasmids tetracycline (Tc) resistant but ampicillin sensitive.
Approximately 1µg of R. vannielii high molecular weight IR DNA was obtained and purified as described in section 3.3.

As S1 nuclease has been reported to leave staggered ends in some cases (Shishido & Ando, 1981), the IR DNA was treated with 0.25 units of T4 DNA Polymerase (BRL) in a buffer containing 33mM Tris/acetate pH 7.9, 66mM potassium acetate, 10mM magnesium acetate, 100µg/ml BSA, 0.5mM DTT, and 0.1mM dATP, dGTP, dCTP, dTTP for five minutes at 37°C. This treatment should "fill in" any terminal single stranded regions. The IR DNA was then deproteinised by extraction with phenol/chloroform/amyl alchohol and chloroform followed by ethanol precipitation. The IR DNA pellet was resuspended in sterile distilled water and was ligated with PstI linkers prepared as follows. About 1µg of PstI molecular linker (BRL) was phosphorylated using 1 unit of T4 polynucleotide kinase in ligase-kinase buffer (66mM Tris/HCl pH 7.6, 10mM $MgCl_2$, 15mM DTT, 200µg/ml BSA, 1mM ATP, 1mM spermidine). After 1h incubation at 37°C, approximately 0.5µg of IR DNA and 1 unit of T4 DNA ligase were added. The ligation was allowed to continue for 4h at 22°C before stopping the reaction by addition of Na_2EDTA to 20mM, deproteinisation as before, and ethanol precipitation. Pelleted IR-linker DNA was resuspended in restriction buffer and digested to completion with PstI. The digested DNA was then separated from unligated linker DNA by gel filtration in a 2ml column of Sephadex G-50. Pooled fractions of void volume were ethanol precipitated and ligated into pBR322. The plasmid vector, pBR322, was linearised by digestion with PstI and dephosphorylated at its 5' ends by treatment with bacterial alkaline phosphatase as described in section 2.20a. Ethanol precipitated vector and insert DNA were resuspended in ligation buffer and mixed to give a 1:1 by weight ratio of

vector:insert (this is equivalent to 1:10 vector:insert in molecules or ends), 0.5 units of T4 DNA ligase were added, and the reaction incubated for 6h at 22°C. Recombinant plasmids were transformed into E. coli K12 strain HB101 and aliquots were plated on selective media to estimate the number of recombinants obtained. Approximately 250 Tc^R transformants were obtained in total, of which about 1/3 were $Ap^S Tc^R$ i.e. containing inserted DNA.

Fifteen randomly selected $Ap^S Tc^R$ clones were inoculated as crosses onto a nitrocellulose filter which had been placed on the surface of a nutrient agar plate containing 15µg/ml Tc. As a control HB101 carrying pBR322 was also plated on the filter. The plate was incubated overnight at 37°C and all of the patches showed good growth. The colonies were lysed in situ by the method of Maniatis et al. (1982) as follows. The nitrocellulose filter, carrying the grown colony patches, was sequentially laid on circles of 3MM filter paper saturated with:

a. 10% SDS - for 3 minutes

b. 0.5M NaOH, 1.5M NaCl - for 5 minutes

c. 3M NaCl, 0.5M Tris/HCl pH 7.0 - for 5 minutes

d. 0.36M NaCl, 0.02M NaH_2PO_4, 0.002M Na_2EDTA pH 7.4 - for 5 minutes

The filter was then air-dried for 60 minutes and baked in a vacuum oven for 2h at 85°C. Hybridization of nick translated high molecular weight IR DNA (10^6 cpm) was carried out as follows (Maniatis et al., 1982). The filter was first washed in 0.05M Tris/HCl pH 8.0, 1M NaCl, 0.001M Na_2EDTA, 0.1% SDS for 1h at 42°C. Prehybridization was then performed in 0.1% (w/v) BSA, ficoll, polyvinylpyrrolidone

and SDS in 3xSSC with 100µg/ml denatured CT-DNA for 2-3h at 68°C. Hybridization was performed in a heat-sealed polythene bag containing prehybridization buffer plus denatured probe DNA for 20h at 68°C.

The filter was washed as follows:

1. Wash at room temperature with 4 changes of 0.1% (w/v) SDS, 2xSSC for 5 minutes each.

2. Wash at 68°C with 2 changes of 0.1% SDS, 1xSSC for 30 minutes each.

3. Wash at 68°C once in 0.1% SDS, 0.1xSSC for 30 minutes.

4.Rinse in H_2O.

The filter was then air-dried and autoradiographed. All of the colonies showed faint hybridization with the high molecular weight IR DNA probe, including that containing pBR322 alone (not shown). This suggests that either the hybridization conditions were not sufficiently stringent, which seems unlikely, or that R. vannielii high molecular weight IR DNA shares homology with some sequences in the E. coli chromosome. The inability to specifically detect inserted IR DNA by colony hybridization or by gel electrophoresis of restricted plasmid DNA (also not shown) led to this series of experiments being terminated. The general conclusion drawn is that cloned IR DNA would be best obtained by hybridization screening of chromosomal gene libraries constructed in a plasmid with no homology with R. vannielii DNA, under conditions which minimize any possible hybridization to E. coli DNA.

3.8 DNA rearrangements in the R. vannielii cell cycle

As discussed in section 1.3, DNA rearrangement represents a widespread mechanism by which relatively stable changes in gene expression may be effected. Such a mechanism may be of special significance in cellular differentiation where some changes in gene expression may need to be stable over long periods of time. DNA rearrangements have been implicated in cellular differentiation in Caulobacter crescentus (Nisen et al., 1979) and Bacillus subtilis (Rhaese et al., 1982). It is possible that DNA rearrangements occur during the cell cycle of R. vannielii and two methods were used to look for such rearrangements.

3.8.1 Dual labelling method

DNA from complex cycle cells and inhibited swarmer cells was labelled in vivo with ^{3}H-adenosine and ^{32}P-orthophosphate respectively, as described in sections 2.13 and 2.12. Tritiated complex cycle DNA and ^{32}P-labelled 0h swarmer cell DNA (10^{4} cpm of each) were mixed with 2.5µg of cold carrier complex cycle DNA and digested with the restriction enzyme EcoRI. The digested DNA was then run in a 0.7% agarose horizontal slab gel with HindIII digested λ DNA as size markers. Individual digest lanes were cut out of the gel and dried onto strips of 3MM filter paper. The dried gel strips were cut into 1mm slices and the radioactivity of each slice estimated by scintillation counting as described in section 2.7c. Overspill between ^{3}H and ^{32}P channels was estimated by use of controls containing ^{3}H or ^{32}P radioactivity alone. The ratio of ^{3}H to ^{32}P

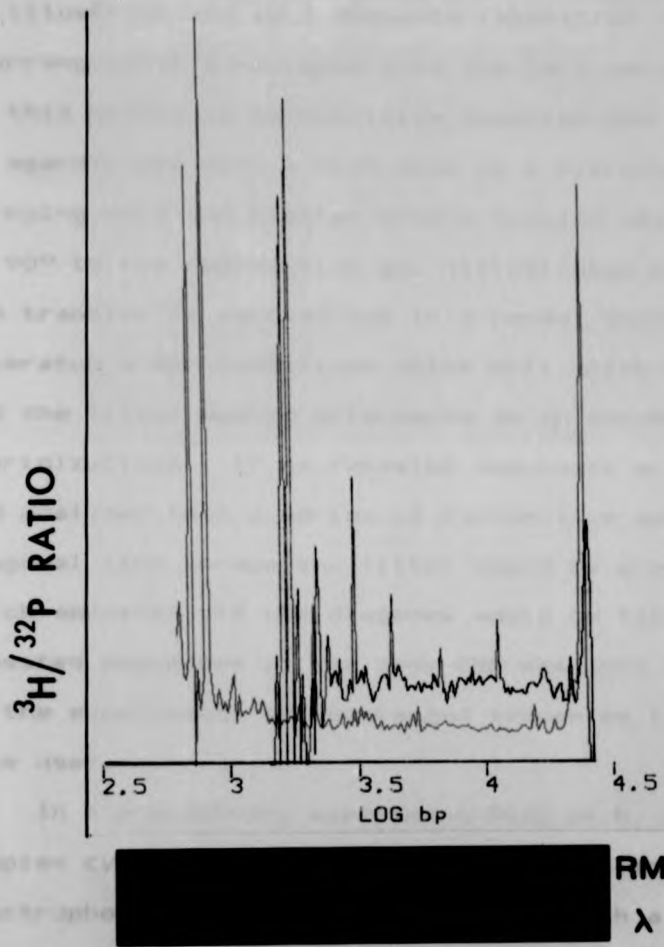
counts in each slice was calculated using the program RATIO (listed in appendix 1) in a PET 8032 microcomputer, taking account of blanks, channels overspill and efficiency of counting for each isotope. The $^{3}H/^{32}P$ ratio for each gel slice was then plotted against the logarithm of the corresponding DNA fragment size using the program RPLOT (listed in appendix 2) in a BBC model B microcomputer. The resulting graphical representation of $^{3}H/^{32}P$ ratio against log fragment size is shown in figure 3.17 for two separate experiments and includes a representative gel track. Statistically significant variations of the $^{3}H/^{32}P$ ratio from the mean value should indicate those bands which are present in one digest and absent from the other, suggesting a possible DNA rearrangement.

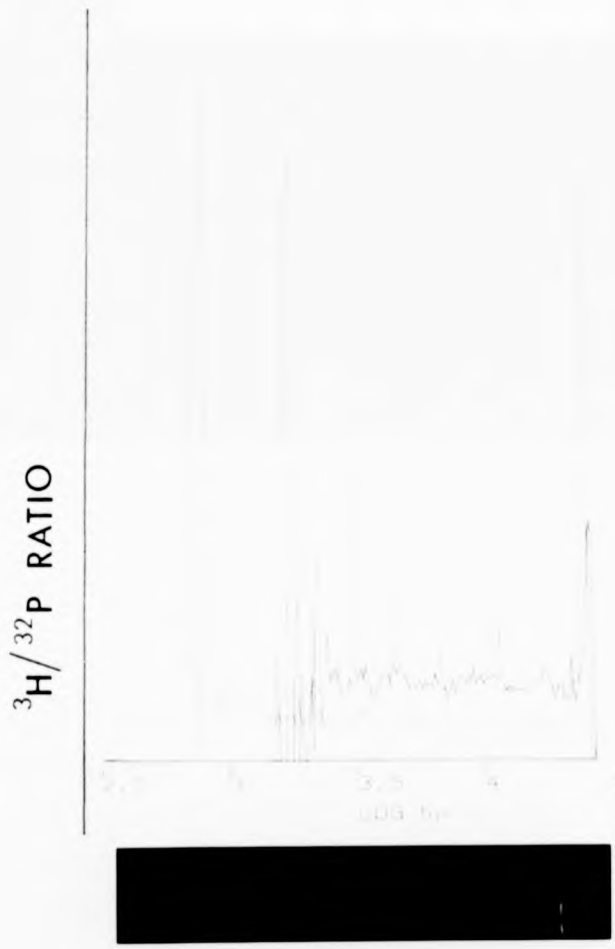
Although both curves in figure 3.17 show a number of significant variations from the mean peak height there is only one correspondence between the gels. This peak is equivalent to a DNA fragment of about 2kb in size and represents the lack of a ^{32}P-labelled band in the 0h swarmer cell digest - possibly due to a DNA rearrangement. The lack of correspondence between the other peaks however, casts doubt on the validity of this result. Further work would be needed before the possibility of a DNA rearrangement could be confirmed, either by the repetition of these experiments or the hybridization of DNA fragments of about 2Kb excised from gels to complex cycle and swarmer cell chromosomal restriction digests.

3.8.2 Crossed-blot hybridization method

The observation that both high and low molecular weight

Figure 3.17 Dual-labelling experiment. $^{3}H/^{32}P$ ratio in each 1mm slice of a 0.7% agarose gel of an EcoRI digestion of mixed ^{3}H-labelled complex cycle and ^{32}P-labelled 0h swarmer cell DNA is plotted against approximate DNA fragment size. Two independent experiments are shown. A representative gel track and HindIII digested λ size standards are shown below. Note that the peaks at the right and left ends of each trace represent the top and bottom respectively of the gels used. It is apparent from this that the gel represented by the blue trace has run considerably further than the other.


$^3H/^{32}P$ RATIO
2.5
3
3.5
4
4.5
LOG bp
RM5/EcoRI
λ


$^{3}H/^{32}P$ RATIO
RM5/EcoRI
λ

IR DNA hybridized to many bands in restriction digests of R. vannielii chromosomal DNA suggested that some of these sequences might be repeated within the R. vannielii genome. The crossed-blot hybridization method was used in an attempt to illustrate any such sequence repetition as well as DNA rearrangements associated with the cell cycle. The strategy of this method is to hybridize labelled DNA fractionated in an agarose gel with a wide slot to a nitrocellulose filter carrying cold DNA blotted from a similar gel, but oriented at 90° to the radioactive gel (illustrated in figure 3.18). DNA transfer is carried out in a normal Southern blotting apparatus under conditions which will allow hybridization and the filter washed afterwards as in conventional hybridizations. If no repeated sequences are present in the DNA analysed then a series of radioactive spots forming a diagonal line across the filter would be expected. Spots which appeared off the diagonal would be taken to indicate repeated sequences if the same DNA was used in both stages of the experiment, or rearranged sequences if different DNAs were used.

In a preliminary experiment 50µg of R. vannielii complex cycle DNA was digested with EcoRI and electrophoresed in a 0.7% agarose gel with a 9cm wide slot. The gel (shown in figure 3.19) was blotted by the standard method (section 2.17). A portion of the digest was retained and labelled with α^{32}P-dGTP as follows:
Digested DNA, in 33mM Tris-acetate pH 7.9, 66mM potassium acetate, 10mM Magnesium acetate, 100µg/ml BSA, 0.5mM DTT, was treated for 3 minutes at 37°C with 0.25 units of T4 DNA Polymerase (BRL). Under these conditions the 3' exonuclease

Figure 3.18 Arrangement of gel and filter used in the criss-cross blotting experiment. This was set up in a standard Southern blotting apparatus at the temperature required.

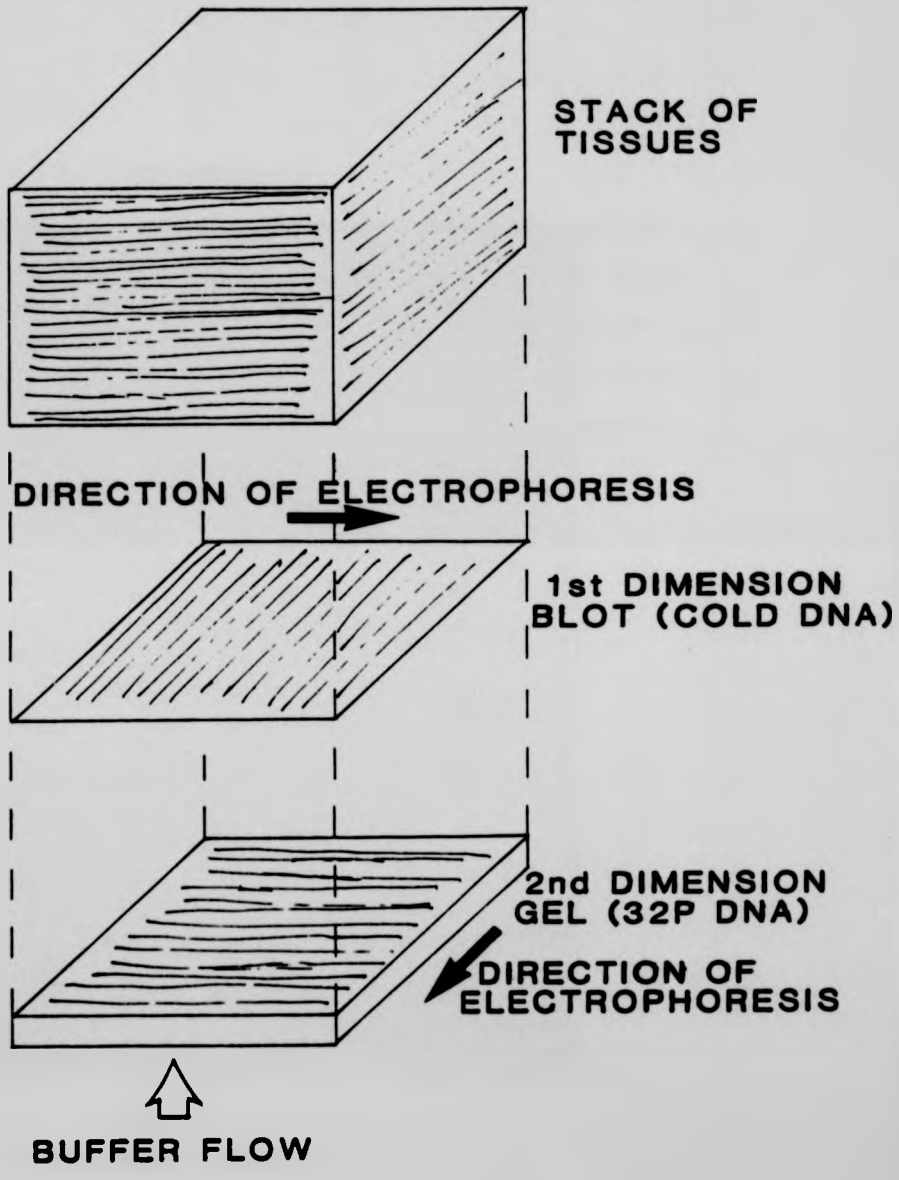

STACK OF TISSUES
DIRECTION OF ELECTROPHORESIS
1st DIMENSION BLOT (COLD DNA)
2nd DIMENSION GEL (32P DNA)
DIRECTION OF ELECTROPHORESIS
BUFFER FLOW

Figure 3.19 EtBr stained 0.7% agarose gel of EcoRI digested R. vannielii DNA run in a gel with a 9cm slot for the first dimension of a criss-cross blot gel. Size standards are HindIII digested λ DNA.

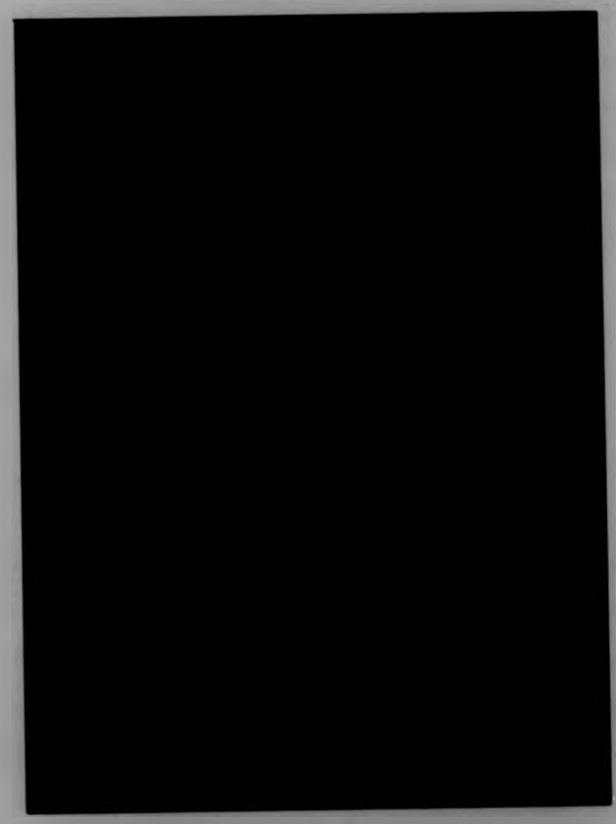
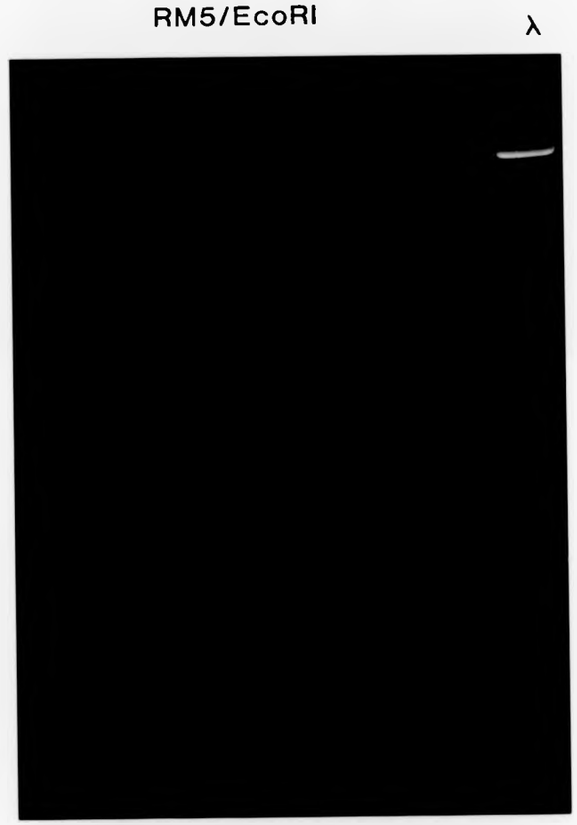


Figure 3.19 EtBr stained 0.7% agarose gel of EcoRI digested R. vannielii DNA run in a gel with a 9cm slot for the first dimension of a criss-cross blot gel. Size standards are HindIII digested λ DNA.



activity of the polymerase removed about 70 bases from the 3' ends of the restriction fragments. On the addition to this of 1µl each of 2mM dATP, dTTP, dCTP and 25µCi of α^{32}P-dGTP the gaps made were filled in by polymerase activity, labelling the DNA fragments to high specific activity. Approximately 10^7 cpm of this EcoRI restricted complex cycle DNA was then run in a 0.7% agarose slab gel with a 9cm slot. The DNA in the gel was denatured, neutralised and assembled in the transfer apparatus as illustrated in figure 3.18. The transfer buffer was 3xSSC and transfer was at 65°C for 16h. On dis-assembly of the apparatus it was found that the majority of the radioactivity had passed through the filter into the stack of tissues above. Autoradiography confirmed that no counts had bound to the filter.

The experiment was repeated using a different set of conditions for transfer. Immediately before transfer the radioactive gel was equilibrated in 10xSSC, 50% (v/v) formamide (deionised) for 1h and transfer was performed at 37°C in this buffer for 16h. In this case too all of the counts were found to pass through the filter. There are several possible reasons for the lack of hybridization found here.

1. The hybridization equilibrium may be sensitive to the flow of liquid through the filter over several hours.
2. The specific activity of the probe DNA may have been insufficient to show up the very small areas expected to exhibit hybridization as spots on autoradiographs.
3. The conditions used for transfer may have been unsuitable for this type of combined blotting and hybridization.

Some of these problems might be solved by reducing the amount of time allowed for transfer and using probe DNA with much higher specific activity.

Similar types of crossed contact hybridization have been described by several groups (Rozek & Timberlake, 1979; Shen & Maniatis, 1980; Wensink et al., 1979). These have used both DNA and RNA gels in restriction mapping of RNA and in the investigation of repeated sequence organisation in eukaryotes. No example of the use of such techniques in the investigation of prokaryotic sequence structure has yet been described, but the potential of this method is considerable.

The experiments described here illustrate some of the difficulties involved in trying to demonstrate DNA rearrangements without using a specific hybridization probe and any future work in this direction should first work towards obtaining such a probe. This may be done either by screening individual clones from chromosomal gene libraries or by investigation of sequences isolated for other reasons e.g. sequences coding for differentially transcribed RNAs; sequences which exhibit specific interaction with proteins; or regions with homology for known translocatable elements.

3.9 Serine proteases in differentiating swarmer cells

In section 1.2.5.3 it was noted that differentiation of *R. vannielii* swarmer cells was accompanied by specific qualitative and quantitative changes in soluble protein. It is likely that these changes are regulated by both protein synthesis and degradation. Thus the intracellular proteases of *R. vannielii* swarmer cells may play a significant role in

differentiation.

The experiment described below represents an initial investigation of the serine proteases of differentiating swarmer cells, using the specific inhibitor di-ispropylfluorophosphate (DFP). DFP covalently interacts with the serine residue at the active site of serine hydrolases, a class of enzymes which includes both serine proteases and some other esterases (Dixon & Webb, 1979). Tritiated DFP was used to specifically label intracellular serine hydrolases at various stages during swarmer cell differentiation and labelled proteins were visualised by fluorography of SDS-polyacrylamide gels.

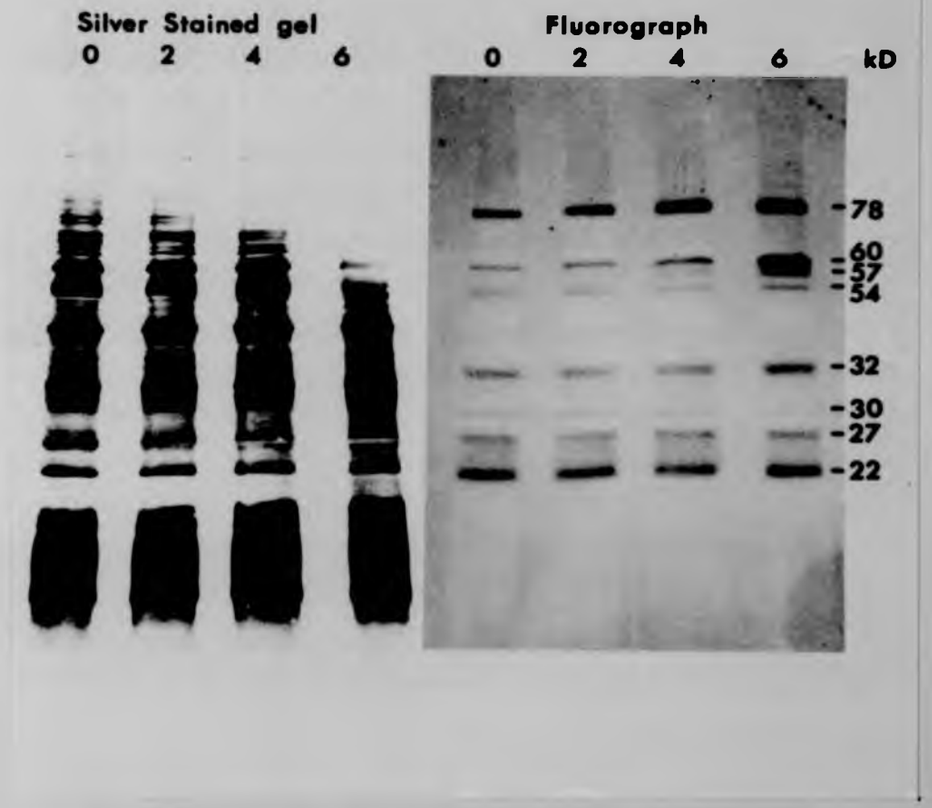
Cells were collected at intervals throughout the differentiation of a synchronous swarmer cell population. For each sample a 40ml aliquot of cells was removed, washed in PM medium and stored at -70°C. When all of the samples had been taken the cells were thawed on ice and resuspended in 1ml PM medium plus 175μCi (35μl) of ^{3}H-DFP (Amersham; specific activity 2Ci/mmol). The cells were incubated at 20°C for 30 minutes, washed three times in PM medium, and resuspended in gel loading buffer (125mM Tris/HCl pH 7.5, 10% (w/v) sucrose, 4% (w/v) SDS, 10mM 2-mercaptoethanol). Proteins were then solubilised by incubating at 100°C for 5 minutes, insoluble material was removed by centrifugation and the supernatants were stored at -20°C. Portions of each supernatant (10^{4} cpm) were electrophoresed in 5-20% SDS-polyacrylamide gradient slab gels according to the method of Laemmli (1970). After electrophoresis gels were fixed by the method of Steck et al. (1980) and stained with silver nitrate by the method of Wray et al. with modifications suggested by Eschenbruch & Burk (1982), as

described in section 2.24. After photography stained gels were soaked twice for 30 minutes in dimethylsuphoxide (DMSO), followed by 3h in 22.2% (w/v) 2,5-diphenyloxazole (PPO) in DMSO. Gels were rinsed for 1h in running water and dried under heat and vacuum. They were then fluorographed for up to 6 months at -70°C.

Figure 3.20 shows a silver stained gel and the corresponding fluorograph of ^{3}H-DFP labelled R. vannielii swarmer cell protein. Only 8 major bands are visualised by DFP labelling in the swarmer cells.

A number of changes in DFP-binding protein profile can be observed during swarmer cell differentiation, the most striking of which is the appearance of a new band of approximately 57kD between 4h and 6h after the initiation of differentiation. Two polypeptides also show increases in concentration during differentiation. The band of about 78kD increases gradually starting at about 2h of differentiation while there is a marked increase in the 60kD band occurring between 4h and 6h. Two minor bands, occurring between the 32kD and 30kD bands, also change in intensity, increasing slightly until about 4h and then disappearing. This variation in the number and concentration of DFP-binding polypeptides during differentiation of R. vannielii swarmer cells may reflect changes in the intracellular concentration of serine proteases. Alternatively, the changes in band intensity may reflect the occupancy of the active sites of these enzymes either by substrate molecules or by intracellular inhibitors. If such is the case then changes in gene expression need not be invoked to account for the changes in banding observed.

Figure 3.20 Silver stain and fluorograph of a 5-20% SDS-polyacrylamide gradient gel of R. vannielii total protein from swarmer cells at 0,2,4 and 6 hours of differentiation, labelled with ^{3}H-Diisopropylfluorophosphate (DFP). Approximate molecular weights for DFP-labelled bands were estimated from the migration of silver stained protein standards (not shown).



Chapter 4 Discussion

4.1. The R. vannielii cell cycle as a system for studying bacterial development

The morphological complexity observed in the cell cycle of Rhodomicrobium vannielii arises from the ability of any reproductive cell to produce on division one of three cell types: mother cell, swarmer cell or exospore, while expression of an alternative, simplified cell cycle may occur apparently in response to changes in growth conditions. Biochemical investigation of the R. vannielii cell cycle is possible only at a limited number of stages, due to the requirement of most biochemical methods for large quantities of cells at the same cell cycle stage. This is only possible for swarmer cells, exospores, and in studies of the complex and simplified cell cycles per se. Swarmer cells and exospores when isolated are synchronized with respect to their cell cycles and are therefore potentially rewarding objects of study. Little work has been done however, on exospore germination (Whittenbury & Dow, 1977), while the differentiation of swarmer cells has been studied extensively.

R. vannielii swarmer cells are highly amenable to research for the following reasons:

1. Homogeneous populations of selectively synchronized swarmer cells may be obtained easily and in large quantities.
2. Selective synchronization minimises perturbation to the swarmer cells.

3. Differentiation of a selected swarmer cell population may be initiated when required and is synchronous over at least 6h.

4. Differentiation may be monitored easily by microscopy or cell volume analysis.

Research is being hindered, however, by the limited amount of genetic background knowledge available; difficulties in obtaining mutants for study; and the lack of a reliable system of genetic exchange in R.vannielii. Most of the present research on the R. vannielii cell cyle and differentiation avoids these difficulties by concentrating on the molecular and biochemical aspects of differentiation (e.g. DNA, RNA, proteins, and their turnover), and by using recombinant DNA techniques.

This project was intended to evaluate the significance of inverted repeat sequence DNA in the differentiation of R. vannielii swarmer cells, with particular emphasis on the possibility of their involvement in DNA rearrangements.

4.2 The structure and function of R. vannielii IR DNA

4.2.1 Synopsis of results

The inverted repeat sequence DNA of R. vannielii was found to comprise approximately 7% of the total chromosomal DNA and was divided into two size classes, each of which was examined independently. The results obtained are summarized below.

1. High molecular weight IR DNA

This class of IR DNA is made up of a heterogeneous

mixture of DNA fragments of 100-700bp in length. It hybridized to many bands in restricted chromosomal DNA and did not appear to highlight any DNA rearrangements during swarmer cell differentiation. The size and genomic distribution of these sequences are similar to those of the families of middle repetitive DNA sequences found in eukaryotic genomes. The archetypal Alu family, found in the DNA of many eukaryotes, makes up approximately 60% of the 300bp fraction of human DNA resistant to S1 nuclease digestion after 0Cot renaturation, and may be isolated either as direct or inverted repeats (Houck et al., 1979). A typical human Alu family member consists of two directly repeated, imperfect copies of a 150bp consensus sequence (Deininger et al., 1981) interspersed between unique sequence regions of 1-2kb throughout the human genome. These repeated sequences have been proposed as possible replication origins or transposable elements (Deininger et al., 1981), but no definite function has yet been assigned to them.

Some component of the high molecular weight IR DNA class appeared to be specifically bound by R. vannielii soluble protein and although quantitative changes in this interaction were observed during swarmer cell differentiation, these results were not sufficiently reproducible to comment on in detail although the general trend appeared to be of an increase in the binding interaction during differentiation. If this increase was an accurate reflection of events in vivo, it would probably represent the activation of differentiation-specific genes by binding of inducer proteins or RNA polymerase. The same

effect may also be produced, however, by an increase in the proportion of non-specific DNA-binding proteins in the cell-free extracts, such as might be expected to occur during DNA replication.

2. Low molecular weight IR DNA

This class of IR DNA is made up of approximately equal quantities of DNA fragments of 17 and 27bp. It hybridized to many bands in EcoRI and HindIII chromosomal digests but only to four bands in an HaeIII digest. The HaeIII hybridization pattern suggests clustering of the low molecular weight IR DNA sequences within fragments of 1620, 1120, 760 and 680bp apparently contradicting the results of hybridization to EcoRI and HindIII digests which imply a dispersed sequence organisation. It is possible, however, to suggest a model which will satify both of these sets of observations. It is proposed that the 17 and 27bp low molecular weight IR DNA fragments form "building blocks" from which the 100-700bp fragments of high molecular weight IR DNA are constructed. The sequential appearance of first high then low molecular weight fractions in the S1 digestion in figure 3.6 and the hybridization of low molecular weight IR DNA throughout the high molecular weight smear in figure 3.14 support this view. The high molecular weight IR DNA fragments are themselves arranged into clusters which produce the four size classes of bands revealed by hybridization to the HaeIII digestion (figure 3.14). These HaeIII fragments are dispersed throughout the genome, demonstrated by the hybridization of both IR DNA fractions to many EcoRI and HindIII restriction fragments, whose

average size is much larger than the HaeIII fragments. This model is consistent with all of the observations of IR DNA hybridization and could be further supported or disproved by hybridization of high molecular weight IR DNA to HaeIII digested chromosomal DNA. The model would predict that in such a hybridization the same four major bands observed in figure 3.14 would be seen. Unfortunately, there was insufficient time available to allow this experiment to be performed.

The hybridization of low molecular weight IR DNA with phage λ DNA (figure 3.12) may be a valid observation as no hybridization to pBR322 DNA was observed under similar conditions. No binding of this IR DNA class to R. vannielii soluble protein could be demonstrated but this may have been due to the small size of the DNA fragments used or to other factors in those assays.

The abundance of IR DNA in R. vannielii is considerably higher than that found in E. coli. In both cases however, discrepancies amounting to 1-2% exist between the results presented here and previously published values based on renaturation experiments (Kato et al., 1974; Potts et al., 1980). These differences may be due to a number of factors.

1. Inter-strain differences

This may account for the results for E. coli but do not explain those for R. vannielii as the same strain was used both here and in the work of Potts (1980).

2. Limited renaturation during cooling

After heat denaturation all samples were immediately cooled on ice. Approximately 1-2 minutes were required for

cooling under the conditions used here and this may have allowed some renaturation of non self-complementary DNA sequences such as directly repeated sequences. This may account for some of the discrepancy in both cases as the same procedure was used throughout.

3. Consistent error in the assay

Although this can not be ruled out it is unlikely to be the source of the discrepancies found. All assays were done at least 3 times with two independent labelled DNA preparations, and positive and negative controls were used in all assays (blank filters, washed filters, undigested DNA and counting controls). The IR DNA obtained was also subjected to a further round of denaturation, 0Cot renaturation and S1 digestion to ensure that precipitable counts were due to duplex, non-crosslinked DNA.

4. Nature of the DNA used

In this work native high molecular weight DNA was used as the substrate for the assays while both Kato et al.(1974) and Potts et al. (1980) used DNA which had been sheared to about 1Kb. It is both possible and likely that the increased fragment size allowed renaturation of sequences which would not be able to renature in sheared DNA molecules. This may account for some of the discrepancy found although the amount would be difficult to quantify without further work.

4.2.2 IR DNA and the cell cycle

A DNA sequence may have an effect on gene expression due to either its nucleotide sequence or its gross structural properties. Inverted repeat sequences have

unique structural and functional characteristics which determine their genetic function - discussed in section 1.3. It is likely that the IR DNA sequences of R. vannielii play a variety of roles in the genome, from being parts of protein coding regions to acting as binding sites for regulatory proteins. Although the experiments described in Chapter 3 determined the abundance and structure of IR DNA, they shed relatively little light on the functional aspects of these sequences. Filter-binding experiments (section 3.6) suggested that about 8% of the high molecular weight IR DNA fraction carried sites for specific binding of proteins, presumably regulatory proteins of some sort. Well characterised binding sites for sequence-specific proteins all appear to be short sequences of about 25bp with at least partial dyad symmetry (Gicquel-Sanzey & Cossart, 1982). The DNA fragments used in the binding studies presented here had a size range of 100-700bp. This implies that if the sequence-specific DNA-protein binding sites were of a similar size and sequence structure to those described above, then the IR DNA sequences would probably possess complex internal repeat structure, part of which would form the protein binding sites. Such a sequence structure could explain the size heterogeneity of the high molecular weight IR DNA fraction.

Further investigation of the nature and function of specific DNA-protein binding would require the isolation of binding sites, probably by recombinant DNA techniques, and possibly the purification of DNA binding proteins. This would allow the investigation of individual binding interactions, the sequences flanking the binding sites, the

and any variations in the abundance of individual binding proteins which might occur during the cell cycle.

In section 1.3.3 a number of genetic processes which involved inverted repeat sequences were described. Although no specific functions can be ascribed to the inverted repeat sequences of R. vannielii it is relatively easy to suggest roles which they might play.

Rearrangement of DNA sequences is now known to be responsible for specific changes in gene expression in a number of cases, in species as diverse as Salmonella typhimurium (Zieg et al., 1978) and Neisseria gonorrheae (Meyer et al., 1982). The changes in gene expression brought about by such rearrangements, in contrast to other regulatory mechanisms, are relatively stable, lasting at least one generation time. In R. vannielii such stable changes in gene expression are expected to occur both cyclically, e.g. during swarmer cell maturation; and over longer periods, e.g. the switch between complex and simplified cell cycles. That such events could be controlled by inversion or translocation of DNA segments involving inverted repeat sequences is a possibility which cannot be ignored. It is also known that homologous recombination between inversely repeated rRNA operons of E. coli leads to their inversion at frequencies of 1% (Hill & Harnish, 1981). If the structure of the rRNA genes of R. vannielii is similar, then the switching on of rRNA synthesis early in swarmer cell differentiation might be due to a programmed rearrangement of the rRNA genes.

The formation of stable stem-loop structures by inverted repeat sequences was discussed in section 1.3.3.4

inverted repeat sequences was discussed in section 1.3.3.4 but little was said about the effects of such structures on gene expression. Although the direct consequences of cruciform formation on gene expression may be expected to be generally disruptive, secondary effects may also occur due to the influence of stem-loop structures on tertiary and quaternary DNA structure. The presence of such structures in substrate DNA was found to inhibit its packaging into reconstituted eukaryotic nucleosomal core particles, suggesting that the cruciform structure was incompatible with nucleosome formation (Nickol & Martin, 1983). It seems likely that the presence of cruciform structures in prokaryotic DNA would similarly affect its packing in the bacterial nucleoid and might define which areas of the genome were or were not packaged. The conformation of the nucleoid may play a significant part in control of gene expression by determining the loci which are available for transcription, so that the presence of stem-loop structures at or near specific genes might increase or decrease their transcription. This is particularly relevant to R. vannielii in which changes in nucleoid conformation can be observed during swarmer cell differentiation (Whittenbury & Dow, 1977).

In amino acid biosynthetic operons of E.coli and S. typhimurium the operator and promoter sites are close to a region of multiple inverted repeat structure called the attenuator. The frequency of transcription termination in the attenuator region is modulated by the availability of the appropriate charged tRNA molecules (Yanofsky, 1981). The structure of this region, which is composed of inverted

repeat sequences and sequence-specific DNA-protein binding sites, is similar to that proposed for the protein-binding fraction of R. vannielii IR DNA and suggests a possible function for the multiple repeat structures thought to be present.

If inverted repeat sequences are transcribed, the RNA molecules produced will have strong secondary structure. In Caulobacter crescentus, a bacterium which shows cellular differentiation similar to R. vannielii, an RNA processing enzyme specific for double-stranded RNA has been characterised (Bellofatto et al., 1983). The presence of such an enzyme at defined times in the R. vannielii cell cycle would lead to the production of defined sets of processed RNAs from regions containing inverted repeat sequences, which would not be produced in the absence of the enzyme. This would allow concerted regulation of a large number of transcripts by a relatively simple mechanism.

It is now clear that any investigation of the function of the inverted repeat sequence DNA of Rhodomicrobium vannielii must use as its raw material not heterogeneous total IR DNA preparations but IR DNA which has been fractionated to reduce the number of different fragments involved. This would require cloned IR DNA to provide sufficient quantities of each IR DNA species. The best source of such cloned sequences would be a chromosomal gene library which had been repeatedly screened by hybridization with IR DNA . Such a gene library could be further fractionated on the basis of its transcription, either in differentiating swarmer cells or in complex cultures;

specific protein-binding activity; homology with transposons or IS sequences; or sequence structure e.g. possession of both halves of an inverted repeat sequence. Such fractionated IR DNA libraries could then be used to probe for cell-cycle associated DNA rearrangements, sequences which are differentially transcribed during swarmer cell differentiation, sequence-specific DNA binding proteins whose concentrations change during differentiation and other DNA sequences which might prove important in cell cycle control.

4.3 Genome rearrangements in R. vannielii

In this work only one of the experimental strategies employed to look for genome rearrangements yielded any usable results. These dual label experiments were intended to highlight quantitative differences in restriction digestion patterns between cells at different stages in the cell cycle which are not detected by direct observation of stained gels. The results obtained for EcoRI digested complex cycle and Oh swarmer cell DNA (illustrated in figure 3.17) showed up a number of significant deviations from the mean $^{3}H/^{32}P$ ratio each time the experiment was performed. These deviations however, appeared to correspond in only one case. While the lack of correspondance between the other peaks casts some doubt on these experiments, there is still a case to be made for the validity of the observations.

1. The size of the deviations. For the blue curve the mean ratio in the region of the peak of interest was 0.39 and the

sample standard deviation (SD) was 0.22. The peak ratio was 0.93 at an approximate fragment size of 2.2kb. This is equivalent to a deviation of 2.4 SD above the mean. For the red curve the mean was 2.12, SD was 1.38 and the peak ratio was 6.78 at a fragment size of about 2.1kb. This represents a deviation of 3.4 SD above the mean. The differences in ratio between the two sets of results is due to decay of ^{32}P in the time between the two experiments, leading to a reduction in $^{3}H/^{32}P$ ratio.

2. The relatively small variation observed over the rest of the curves, except at the ends of the gels where such variations might be expected.

3. In similar experiments Hintermann et al. (1982) detected possible rearrangements in the genome of Streptomyces glaucescens. The peak heights reported above are at least comparable with the results of this group, giving further support for their validity.

These results suggest that further experiments of this sort might prove fruitful in the investigation of DNA rearrangements in the R. vannielii cell cycle and also in looking at the long-term stability of the R. vannielii genome. Such experiments might also be of some use in molecular taxonomic studies, giving a measure of the similarities between restriction digests of DNA from different strains or species. Its value would be limited however, to closely related bacterial species.

The crossed-blot hybridisation method is also, in theory, a powerful tool for the visualisation of repeated sequences in a single type of DNA, and of rearranged and repeated sequences in DNA from different sources. The lack

of results obtained here, which is ascribed mainly to insufficient specific activity in the the probe DNA, does not detract from the potential usefulness of the technique.

4.4 Serine proteases in differentiating swarmer cells

The importance of serine proteases in the differentiation of *R. vannielii* swarmer cells has not yet been determined, but the results presented here (figure 3.20) suggest that some serine proteases may have a role late in differentiation, possibly associated with cell division. In *Escherichia coli* the ATP-dependent serine protease La (Swamy & Goldberg, 1982) has been implicated in control of cell division, sensitivity to ultraviolet light and the degradation of DNA-binding proteins (Chung & Goldberg, 1982). The activity of this protease, the product of the *lon* gene, is known to control the stability of the *sulA* gene product, a putative inhibitor of cell division which is involved in the SOS response to DNA damage (Mizusawa & Gottesman, 1983). It is thought that protease La, under normal conditions, keeps the concentration of the sulA protein below that required to inhibit cell division. When the SOS response is stimulated by DNA damage, protease La is inactivated or degraded. This allows the sulA protein to build up and inhibit cell division until DNA repair has been effected. When the SOS response is over, protease La degrades the excess sulA protein, allowing cell division to resume. It is tempting to speculate that some of the proteases in *R. vannielii* swarmer cells may have a similar

role in control of cell division, particularly those which appear or increase late in differentiation like the 60kD and 57kD bands in figure 3.20. Further work in this field will be aimed at identifying those DFP-labelled bands which are proteases and defining the role of serine proteases in swarmer cell differentiation, initially using specific inhibitors and subsequently by purification of the various proteases. The DFP-labelling technique will prove useful in this work, facilitating the purification of the proteins by allowing their detection and identification in heterogeneous protein mixtures.

4.5 Future prospects for analysis of the R. vannielii cell cycle

4.5.1 Introduction

Most of the present research on R. vannielii is concerned with the regulation of its cell cycle. The practical aim of this research is to determine the nature of the events in the R. vannielii cell cycle and to elucidate the molecular mechanisms by which they are controlled. This goal can only be attained by painstaking investigation of the many processes which are involved in the cell cycle. The investigation of these processes represents the short-term priorities on which individual research projects are based. There are several such projects presently under way which are concerned with a number of aspects of swarmer cell differentiation.

1. Composition and template specificity of RNA polymerase.
2. Structure and expression of rRNA genes.

3. DNA rearrangements.
4. Sequence-specific DNA-binding proteins.
5. Protein degradation and modification
6. Membrane-associated proteins
7. Cloning of differentially expressed genes
8. Polyadenylated RNA
9. Temporal control of protein synthesis.
10. Nucleoid conformation

One important area which does not appear to be under investigation is the genetic analysis of R. vannielii and its cell cycle. This area of research has two prerequisites for practical investigation:

1. A method of obtaining reasonable numbers of mutants of many types for use in constructing a genetic map.
2. A system of genetic exchange to allow mapping by complementation and recombination methods and to allow testing/mapping of cloned sequences.

The reasons why such work is not being done are that R. vannielii appears to be refractory to many of the conventional mutagenic methods and that no reliable system of genetic exchange is yet available. R. vannielii has no known bacteriophages or plasmids and attempts to stably introduce broad host range plasmids into R. vannielii have so far been largely unsuccessful. Despite these drawbacks the need for genetic background knowledge must be satisfied before any significant progress can be made in the molecular genetics of the R. vannielii cell cycle.

4.5.2 Experimental approaches to the investigation of *R. vannielii* swarmer cell differentiation

Taking into account the advantages and disadvantages of research on *R. vannielii* swarmer cell differentiation, a number of approaches are open which will allow its investigation at the molecular level. In this section I will outline a few lines of research which might be used in the isolation and analysis of genes important in the differentiation process.

1. Cloning of developmentally regulated genes.

Radioactively labelled total RNA could be used to identify and select those clones in a gene library which contained transcribed DNA sequences. Plasmid DNA from these individual clones could then be screened by duplicate dot hybridisation with labelled RNA from swarmer cells at various stages in differentiation. Sequences which showed developmental regulation could then be analysed by restriction mapping, subcloning, hybridization to chromosomal restriction digests, *in vitro* transcription and translation, minicell and maxicell techniques, promoter mapping by RNA polymerase binding and sequence analysis. This should provide information about the expression of the gene and possibly the properties of the protein product.

2. Cloning of sequences bound specifically by protein.

DNA sequences involved in the control of gene expression tend to be specifically bound by certain proteins e.g. inducers, repressors, RNA polymerase. Such sequences may be enriched by equilibrium binding of DNA and protein in the presence of excess, nonhomologous, competing DNA and recovery of DNA-protein complexes on nitrocellulose filters.

This method can be used to enrich for specifically bound sequences in a R. vannielii gene library by transformation of the plasmid DNA bound by R. vannielii protein into E. coli and preparation of an enriched gene library. Successive rounds of DNA-protein binding and transformation will increase the degree of enrichment observed. This method may be used to prepare gene libraries highly enriched for specifically bound sequences using protein from various stages in swarmer cell differentiation. Comparison of such stage-specific gene libraries may allow direct identification of sequences which show changes in protein binding during differentiation. Alternatively, stage-specific RNA may then be used to screen for differentially transcribed sequences. As well as using the analytical techniques described above, sequences purified in this way might be used for the purification and identification of their specific binding proteins.

3. Isolation of genes for known proteins

A number of differentially synthesized R. vannielii proteins, notably those of the flagellum, can be isolated in sufficient quantities to allow antisera to be raised against them. Such specific antisera can be useful tools in the cloning of the genes for the protein antigen. Two different approaches are possible:

a. Antiserum may be used to detect the protein of interest as the product of cells containing cloned DNA by immunoprecipitation on plates with antiserum in the agar. Alternatively and more sensitively, lysed recombinant colonies or plaques may be immobilised by transfer to nitrocellulose membranes and visualised by

binding of specific antibody followed by a labelled, non-specific second antibody. In either case only those genes which can be expressed when cloned will be detectable.
b. Another way to use a specific antiserum is to immunoprecipitate polysomes from cells synthesizing the protein of interest. RNA from such polysomes should be highly enriched for gene sequences and may be used directly as a probe to detect the cloned sequence or as a template for cDNA synthesis. The cDNA clones produced may then be analysed and individually used as probes to screen gene libraries of larger DNA fragments.

This method was used to isolate cloned sequences corresponding to the flagellin genes of Caulobacter crescentus (Milhausen et al., 1982)

Analysis of genes isolated by either of these methods may by done by all of the techniques described in section 1 above.

4. Use of transposons

Assuming that a suitable vector for genetic exchange in R. vannielii is found or developed, transposons will be invaluable in the genetic analysis of swarmer cell differentiation. Transposons have two main properties which make them useful in this type of work. First, their ability to insert at many sites in DNA molecules and second, the expression of a phenotype (usually drug resistance) at that site.

Transposons could be inserted into the R. vannielii chromosome by the use of unstable "suicide" plasmids. This would be useful in generating mutants and the drug

resistance markers could be used to help map transposon-induced mutations. Site-directed transposon mutagenesis of cloned R. vannielii sequences would also be possible. Returning such mutagenised genes to R. vannielii cells on stable broad host range plasmids might provide useful information about the normal expression of the cloned gene and its involvement in the cell cycle.

4.6 Conclusion

The aims of this project were the physical characterization of the IR DNA of R. vannielii; the determination of the patterns of its hybridization with chromosomal DNA; the detection of any changes in these patterns which might occur during swarmer cell differentiation and the characterization through recombinant DNA techniques of the DNA fragments involved in such changes. Of these objectives most were achieved, but no changes in hybridization pattern were detected and IR DNA fragments proved difficult to clone by direct means. The characterization of the IR DNA was carried a step further in the protein binding experiments which detected a specific interaction between a fraction of about 8% of the IR DNA and soluble R. vannielii protein. Further work was done in the investigation of cell cycle-associated DNA rearrangements where some evidence for the rearrangement of a 2kb EcoRI fragment was found. Initial experiments were also performed in the investigation of the involvement of serine proteases in swarmer cell differentiation which suggested that some of these proteases might be important in late events, possibly

cell division.

The results presented in this thesis should provide a usable basis and guide for further work on the molecular biology of the R. vannielii cell cycle. In particular, work involving IR DNA, DNA rearrangements or the involvement of serine proteases in differentiation should benefit. Some of the methods described here may also prove useful in further work, particularly those which were specially developed or adapted for use with R. vannielii.

Appendix 1: Commodore CBM microcomputer program "RATIO"

This program was written to correct ^{3}H and ^{32}P counts from an LkB Minibeta liquid scintillation counter to take account of blanks and channels overspill and to calculate the $^{3}H/^{32}P$ ratio of the corrected counts.

```
 10 PRINT "DUAL-LABEL EXPERIMENT"
 20 PRINT"COUNTS RATIO PROGRAM"
 25 DIMH(200),P(200),R(200),HC(200),PC(200)
 30 PRINT"DO YOU WANT DATA RETREIVAL?"
 35 INPUT M$: IFM$="" GOTO35
 40 IFM$="YES" THEN GOSUB4000: GOTO250
 45 INPUT"FILE NAME?"; F$
 50 INPUT"NUMBER OF ENTRIES?"; I
 60 PRINT"ENTER 3H CPM & 32P CPM FOR EACH SAMPLE"
 65 PRINT"CORRECTIONS MAY BE MADE AT THE END"
 70 FOR N=1TOI: PRINT"3H CPM";N;: GOSUB5100: H(N)=VAL(J$)
 75 PRINTTAB(40)"32P CPM";N;: GOSUB5100: P(N)=VAL(J$):
    NEXTN
 80 GOSUB500: PRINTTAB(10)"DO ANY CORRECTIONS NEED TO BE
    MADE? (Y/N)"
 90 GETA$: IF A$="" GOTO90
100 IF A$="N" GOTO160
110 INPUT"ENTRY NUMBER?";X
120 INPUT"3H CPM, 32P CPM?";H(X), P(X)
130 GOTO 80
150 REM****** 32P BACKGROUND CORRECTION ******
160 INPUT"32P BACKGROUND CPM?"; BP
```

```
170 FOR N=1TOI: PC(N)=P(N)-BP: NEXTN
180 REM****** 3H CORRECTION FOR 32P OVERSPILL ******
190 INPUT"3H CORRECTION FACTOR?"; F
200 INPUT"3H BACKGROUND CPM?"; BH
210 FOR N=1TOI: HC=(H(N)-F*PC(N)-BH) *10/6: NEXTN
220 REM****** CALCULATE RATIO ******
230 FOR N=1TOI: IF PC(N) <=0 THEN 245
235 IF HC(N) <=0 THEN 245
240 R(N)= HC(N)/PC(N): NEXTN: GOTO250
245 R(N)=0: NEXTN
250 PRINT"SELECT:"
255 PRINTTAB(20)"SCREEN DISPLAY DATA"
260 PRINTTAB(20)"DISC STORAGE"
270 PRINTTAB(20)"RETREIVE DATA FROM DISC"
280 PRINTTAB(20)"PRINT OUT DATA"
290 PRINTTAB(20)"END"
300 GETB$: IF B$="" GOTO300
310 IF B$="S" GOSUB1000
320 IF B$="D" GOSUB2000
330 IF B$="P" GOSUB3000
340 IF B$="R" GOSUB4000
350 IF B$="E" GOTO9999
360 GOTO250
500 PRINT"3H CPM";: PRINTTAB(30)"32P CPM"
510 FOR N=1TOI: PRINT"H(";N;")=";H(N)
520 PRINTTAB(30)"P(";N;")=";P(N): NEXTN
530 GOSUB5000: PRINT"SELECT:"
540 PRINTTAB(40)"1. REPEAT"
550 PRINTTAB(40)"2. CONTINUE"
560 GETZ$: IF Z$="" THEN560
```

```
 570 IF VAL(Z$)=1 THEN500: IF VAL(Z$)=2 THEN580
 580 RETURN
1000 PRINT"3H CPM"; PRINTTAB(30)"32P CPM";
     PRINTTAB(60)"RATIO 3H/32P"
1010 FOR N=1TOI: PRINT"H(";N;")="; HC(N)
1020 PRINTTAB(30)"P(";N;")="; PC(N);
     PRINTTAB(60)"R(";N;")="; R(N): NEXTN
1030 GOSUB5000
1040 RETURN
2000 REM ****** DISC STORAGE ******
2010 PRINT"ENSURE THERE IS A DISC IN DRIVE O"
2020 PRINT"TYPE C TO CONTINUE"
2030 GETL$: IF L$="" GOTO2030
2040 PRINT"ENSURE DRIVE IS ENGAGED !"
2050 Y$= "O:" +F$+ "H,SEQ,W"
2060 CLOSE1: OPEN1,8,2,Y$
2070 FOR N=1TOI
2080 PRINT#1, HC(N); CHR$(13); NEXTN: CLOSE1
2090 X$= "O:" +F$+ "P,SEQ,W"
2100 CLOSE2: OPEN2,8,2,X$
2110 FOR N=1TOI
2120 PRINT#2, PC(N); CHR$(13); NEXTN: CLOSE2
2130 W$= "O:" +F$+ "R,SEQ,W"
2140 CLOSE3: OPEN3,8,2,W$
2150 FOR N=1TOI
2160 PRINT#3, R(N); CHR$(13); NEXTN: CLOSE3
2170 RETURN
3000 REM ****** DATA PRINT OUT ******
3010 CLOSE1: CLOSE2: CLOSE3: OPEN3,4: PRINT#3
3020 CMD3, "NUMBER"; SPC(7); "3H CPM"; SPC(13); "32P CPM";
```

```
    SPC(15); "RATIO 3H/32P"
3030 PRINT#3: CLOSE3: OPEN2,4,2: OPEN1,4,1
3040 F$="999    99999.99   99999.99   9999.99"
3050 PRINT#2,F$: FOR N=1TOI
3060 PRINT#1,N,HC(N),PC(N),R(N): NEXTN: CLOSE1: CLOSE2
3070 RETURN
4000 REM ****** DATA RETREIVAL ******
4010 PRINTTAB(20)"DATA RETREIVAL"
4020 INPUT"FILE NAME TO BE RETREIVED ?"; V$
4030 INPUT"NUMBER OF ENTRIES ?"; I
4040 U$="0:" +V$+ "H,SEQ,R"
4050 T$="0:" +V$+ "P,SEQ,R"
4060 S$="0:" +V$+ "R,SEQ,R"
4070 CLOSE1: OPEN1,8,2,U$
4080 FOR N=1TOI
4090 INPUT#1, HC(N): PRINT"H(";N;")="; HC(N): NEXTN: CLOSE1
4100 CLOSE2: OPEN2,8,2,T$
4110 FOR N=1TOI
4120 INPUT#2, PC(N): NEXTN: CLOSE2
4130 CLOSE3: OPEN3,8,2,S$
4140 FOR N=1TOI
4150 INPUT#3, R(N): NEXTN: CLOSE3
4160 PRINT"DATA RETREIVAL COMPLETE"
4170 Q=0: FOR N=1TO1000: Q=Q+N: NEXTN: RETURN
5000 PRINT"TYPE C TO CONTINUE"
5010 GETM$: IFM$="" GOTO 5010
5020 RETURN
5100 J$=""
5110 A$="": GETA$: IF A$="" THEN5110
5120 IF A$="." THEN5150
```

```
5130 IF VAL(A$) <0 OR VAL(A$) >9 THEN5110
5140 J$= J$+A$: GOTO5110
5150 PRINT VAL(J$): RETURN
9999 END
```

Appendix 2: BBC Microcomputer program "RPLOT"

This program was written to use the ratios calculated in the program "RATIO" (listed in appendix 1), converting them into a plot of ratio against approximate fragment size caculated from DNA size standards. The final graph is plotted on a logarithmic scale to make it easier to relate to the original gel.

```
 10 DIMA(200),S(10),T(19),M(10),K(200),B(200)
 20 INPUT"DO YOU WANT TO ENTER NEW DATA (Y/N)",A$
 30 IF A$="Y" PROCINPUT ELSEGOTO40
 40 CLS:INPUT"FILE NAME";F$
 50 X=OPENIN F$
 60 F=1:REPEAT
 70 INPUT#X,A(F):F=F+1
 80 UNTILEOF#X
 90 CLOSE#X
100 FORN=1TOF-1:M=164-N
110 B(M)=A(N):NEXT
120 PROCLAMBDA
130 DATA23700,9460,6610,4260,2260,1980
140 MODE4:VDU19,0,4,0,0,0
150 CLS:PRINT TAB(10,5)"1.DISPLAY RESULTS"
160 PRINT TAB(10,10)"2.HARD COPY RESULTS"
170 PRINT TAB(10,15)"3.DISPLAY GRAPH"
180 PRINT TAB(10,20)"4.HARD COPY GRAPH"
190 PRINT TAB(10,25)"5.END"
200 REPEAT
210 V$=GET$
```

```
220 UNTILVAL(V$)<6ANDVAL(V$)>0
230 ONVAL(V$)GOTO240,290,340,370,400
240 CLS:VDU14:PRINT"HIT SHIFT KEY FOR NEXT PAGE"
250 FORN=1TO500:NEXTN
260 PROCDISP
270 PROCWAIT
280 VDU15:GOTO150
290 CLS
300 VDU2
310 PROCDISP
320 VDU3
330 GOTO150
340 PROCGRAPH
350 PROCWAIT
360 GOTO140
370 PROCPLOT
380 PROCWAIT
390 GOTO140
400 MODE4:END
410 DEF PROCLAMBDA
420 PRINT"ENTER MIGRATION IN MM OF THE"
430 PRINT"LAMBDA STDS  OF SIZES GIVEN"
440 Z=1:REPEAT
450 READL:S(Z)=L:PRINT
460 PRINT;S(Z);"BP FRAGMENT";
470 INPUT TAB(20)M(Z):Z=Z+1
480 UNTILZ=7
490 FORZ=1TO6:B=B+M(Z):D=D+(M(Z)^2)
500 T(Z)=LOG(S(Z)):C=C+T(Z):E=E+(T(Z)^2)
510 G=G+M(Z)*T(Z):NEXT
```

```
520 S=(6*G-C*B)/(6*D-B^2)
530 I=(C-S*B)/6
540 CLS:PRINT"BEST LINE IS Y=";I;"+";S;"*X"
550 B=S*(G-B*C/6):E=E-C^2/6:C=E-B
560 G=B/E:PRINT"COEFF. OF DETERMINATION=";G
570 PRINT"COEFF OF CORRELATION=";SQRG
580 PRINT"STD. ERROR OF EST.=";SQR(G/4)
590 FORN=163-F TO164:K(N)=I+S*N:NEXT
600 PROCWAIT:ENDPROC
610 DEFPROCDISP
620 @%=&0002020A
630 PRINT TAB(3)"MIGRATION"; TAB(16)"RATIO";
    TAB(26)"LOG(BP)"
640 PRINT:FORN=1TO500:NEXTN
650 FORN=163-F TO164
660 PRINTN,B(N)/100,K(N):NEXT
670 ENDPROC
680 DEFPROCWAIT
690 PRINT TAB(5,30)"HIT SPACEBAR TO CONTINUE"
700 X=GET:IFX=&20THEN710ELSE700
710 ENDPROC
720 DEFPROCGRAPH
730 CLS:M=163-F:MOVE((K(M)*400)-((K(163)-.3)*400)),
    (B(M)*2+100)
740 FORM=164-F TO164: X=((K(M)*400)-((K(163)-.3)*400)):
    Y=(B(M)*2+100)
750 DRAWX,Y
760 NEXT:VDU23;8202;0;0;0
770 ENDPROC
780 DEFPROCINPUT
```

```
 790 INPUT"FILE NAME";N$
 800 INPUT"NUMBER OF ENTRIES";E
 810 N=1:REPEAT
 820 PRINT"RATIO";N;:INPUTA(N)
 830 N=N+1
 840 UNTILN=E+1
 850 PRINT"ARE ANY CORRECTIONS NEEDED (Y/N)"
 860 INPUTB$:IFB$="N"GOTO890
 870 INPUT"ENTRY NUMBER";X;
 880 PRINT"RATIO";X;:INPUTA(X):GOTO850
 890 A=OPENOUT(N$)
 900 FORN=1TOE
 910 PRINT#A,A(N):NEXT
 920 CLOSE#A:ENDPROC
 930 DEF PROCPLOT
 940 VDU2
 950 VDU1,18,1,65,1,13,1,18,1,13
 960 PROCdraw("M",0,-500)
 970 VDU1,73,1,13:REM SET ORIGIN
 980 VDU1,ASC"C",1,ASC"1",1,13
 990 FOR I%=163-F TO 164
1000 X%=K(I%)*200-500
1010 Y%=B(I%)*2
1020 PROCdraw("D",X%,Y%)
1030 NEXT:FORV%=5TO9
1040 U%=V%/2*200-500
1050 PROCdraw("M",U%,0)
1060 PROCdraw("D",U%,-10)
1070 PROCdraw("M",U%,-25)
1080 P$=STR$(V%/2):PROClabel(P$)
```

```
1090 NEXT:PROCdraw("M",200,-50)
1100 R$="LOG bp":PROClabel(R$)
1110 PROCdraw("M",0,500)
1120 VDU3:ENDPROC
1130 DEFPROCdraw(Q$,X%,Y%)
1140 LOCAL I%
1150 Q$=Q$+STR$(X%)+","+STR$(Y%)
1160 FORI%=1TOLEN(Q$)
1170 VDU1,ASC(MID$(Q$,I%,1))
1180 NEXT:VDU1,13
1190 ENDPROC
1200 DEFPROClabel(Q$)
1210 LOCAL I%:L$="P"+Q$
1220 FORI%=1TOLEN(L$)
1230 VDU1,ASC(MID$(L$,I%,1))
1240 NEXT:VDU1,13
1250 ENDPROC
```

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